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(54) Title: MALARIA VACCINE BASED UPON THE ADDITION OF A MSA1 PEPTIDE

(57) Abstract

The present invention relates to a malaria chimeric peptide comprising a vaccinia virus system which expresses a protein corresponding substantially to the major merozoite surface antigen 1 (MSA1) of Plasmodium flaciparum or an immunogenic portion, thereof. In preferred embodiments, the MSA1 peptide is a 593 amino acid peptide corresponding to amino acids 1047 to 1640 of MSA1 and is combined with a signal peptide and/or an anchor peptide. Chimeric peptides having both signal and anchor sequences in combination with MSA1 sequences were the most effective in eliciting an immunogenic response from a vaccinated host.

Malaria Vaccine Based Upon the Addition of a MSA1 Peptide

Field of the Invention

The present invention relates to novel DNA constructs comprising a vector linked to a DNA segment which encodes a protein containing a signal protein at its N-terminus and an anchor sequence at its C-terminus.

More particularly, the present invention relates to vaccines which are useful for the prevention and treatment of malaria caused by Plasmodium falciparum in humans.

Background of the Invention

Preventing or treating malaria has long been a challenging health problem, particularly in developing countries, and the rapid development of drug resistance in the parasite has enhanced the need for the development of a malaria vaccine. Although there has been steady progress over the last decade, several problems still must be overcome, including selection of an appropriate delivery system vehicle and antigen carrier.

Although malaria was believed to have subsided after World War II, recent outbreaks suggest that the disease is on the rise. Malaria is again the leading cause of morbidity/mortality globally and presents an increasing threat in at risk environments. Estimates are that 300 million new cases of malaria occur each year, with mortality of approximately 1% of infected individuals. Prophylactic medications used to prevent the disease have been rendered ineffective by the emergence of drug-resistant strains of the parasite worldwide. Complete vector protection is simply not possible and all attempts to eradicate the relevant species of mosquito have failed.

Four species of protozoa of the genus Plasmodium are

found in man. The four species include: Plasmodium vivax, Plasmodium malariae, Plasmodium falciparum and Plasmodium ovale. Of these, Plasmodium falciparum produces the most pathogenic of the malarias and often results in death. It is responsible for about half of the human cases of malaria found worldwide.

In malaria, the disease is such that infection followed by recovery does not confer meaningful protection to the individual despite a significant antibody response to several of the parasite proteins. The conventional wisdom has been that the parasite either does not possess antigens suitable for the development of a protective response or has evolved mechanisms which allow it to escape the host immune system. Because recent evidence has shown that immune protection is possible using irradiated sporozoites, the latter hypothesis described above is the more reasonable explanation.

The life cycle of the malaria parasite provides several stages at which interference could lead to cessation of the infective process. Included among these stages is the invasion of the erythrocyte by the merozoite. The merozoite represents a potentially attractive target (and perhaps the only target) from which a vaccine may be produced, because the free merozoite, although it has a limited lifetime (one to two hours) occurs earlier in the life cycle of malaria, and the emergence of later stage sexual forms, which are responsible for transmission of the disease, depends upon the erythrocytic stage.

The general life cycle of malaria parasites is the same for human and other animal malaria parasites, thus allowing model studies to be conducted on a rodent species with accurate translation to the human parasite. For example, the rodent malaria parasite strain Plasmodium berghei Anka has a pathology very similar to the FCR-3 strain of Plasmodium falciparum (a well studied variant of the human parasite). In addition, the blood stage of the human parasite can be grown in the laboratory (in human red cells) thus affording a system

for studying the effects of antibodies/inhibitors on the inva-
sion process, and the erythrocytic phase.

In the life cycle of the malaria parasite, a human
becomes infected with malaria from the bite of a female
Anopheles mosquito. The mosquito inserts its probe into a
host and in so doing, injects a sporozoite form of Plasmodium
falciparum, present in the saliva of the mosquito.

The sporozoites which have been injected into the
human host are cleared into a number of host tissue cells,
including liver parenchyma cells (hepatocytes) and macro-
phages. This phase is known as the exoerythrocytic cycle
because at this point in the life cycle the organism has not
yet entered red blood cells. After entering hepatocytes,
sporozoites undergo a transformation into trophozoites, which
incubate and undergo schizogony, rupture and liberate tissue
merozoites. This process takes approximately 7-10 days and,
depending upon species, may repeat itself several times, dur-
ing which time the host feels no effects. In Plasmodium fal-
ciparum, this repetition does not occur. After the incubation
period, the liver or other tissue cells burst open and release
numerous merozoites into the bloodstream.

Shortly thereafter, certain of these blood borne
merozoites invade red blood cells, where they enter the
erythrocytic phase of the life cycle. Within the red blood
cells, young plasmodia have a red nucleus and a ring-shaped,
blue cytoplasm. The plasmodium divides into merozoites, which
may break out of the red blood cell, enter other erythrocytes
and repeat the multiplication process. This period lasts
approximately 48 hours.

During this same 48 hour period of the erythrocytic
cycle, male and female gametocytes are formed in the red blood
cells. These gametocytes also burst out of the red blood
cells along with the merozoites. It is during this period
that the human host experiences the symptoms associated with
malaria. The merozoites which burst forth from the red blood

cells live for only a few hours in the bloodstream. The
gametocytes live for several days or more in the host's blood-
stream.

     The gametocytes are capable of mating only in the
mosquito. Thus, in order for Plasmodium falciparium to pro-
duce sporozoites for infecting a second human host, a mosquito
must first bite a human host carrying gametocytes. These
gametocytes mature into macrogametes, mate in the mosquito's
stomach and produce a zygote. The zygote (ookinete) is active
and moves through the stomach or the midgut wall. Under the
lining of the gut, the ookinete becomes rounded and forms a
cyst called an oocyst, in which hundreds of sporozoites
develop. Sporozoites thereafter invade the entire mosquito
and many of them enter the salivary glands where they are in a
favorable position to infect the next host when the mosquito
feeds on its blood. The life cycle thereafter simply repeats
itself in another human host.

     During the life cycle of Plasmodium falciparium,
inhibition of invasion of the erythrocyte by the merozoite may
be a key to developing an effective vaccine for malaria. Once
the parasite has gained entry into the red cell, exposure to
the immune system is gone.

     In the past, live vaccinia virus was used as a vaccine
to eradicate smallpox successfully, and a recombinant vaccinia
virus expressing viral antigens has been shown to induce a
strong antibody response in immunized animals, conferring pro-
tection against disease (Arita, I., Nature, 1979, 279, 293-
298). Furthermore, it has been shown in animal models that
co-presentation of potential immunogens with highly
immunogenic vaccinia virus proteins can elicit a strong immune
response against that specific immunogen (Moss and Flexner,
Annals of the New York Academy of Sciences, 86-103; Mackett
and Smith, J. Gen. Virol., 1986, 67, 2067-2082; Houard, et
al., J. Gen. Virol., 1995, 76, 421-423; Fujii, et al., J. Gen.
Virol., 1994, 75, 1339-1344; Rodrigues, et al. J. Immunol.,
1994, 153, 4636-4648). Therefore, the utilization of live

recombinant vaccinia virus as a vaccine might overcome many
problems of antigen expression and delivery presently
encountered in the preparation of recombinant proteins in E.
coli, yeast or insect expression systems. A panel of transfer
vectors have been constructed that allow insertion of foreign
genes into several sites within the 180kb vaccinia virus
genome (Earl and Moss, Current Protocols in Molecular Biology,
1993, 16.17.1-16.17.16). Also, it has been reported that
>25kb of foreign DNA can be inserted into the vaccinia virus
genome (Smith and Moss, Gene, 1983, 25, 21-28). The correct
processing (Chakrabarta, et al., Nature, 1986, 320, 535-537)
and the appropriate post-translational modification (Hu, et
al., Nature, 1986, 320, 537-540; Ball, et al., Proc. Natl.
Acad. Sci. USA, 1986, 83, 246-250; de la Salle, et al.,
Nature, 1985, 316, 268-270), transport and secretion (Ball, et
al., Proc. Natl. Acad. Scienc. USA, 1986, 83, 246-250 and
Langford, et al., Mol. Cell. Biol. 1986, 6, 3191-3199) are
dictated by the primary structure of the expressed protein. In
addition, a recombinant vaccinia virus vaccine has the
advantage of being relatively inexpensive and easily stored,
transported and delivered, features which are particularly
important in the developing countries where malaria is most
prevalent.

        Proteins on the surface of merozoites are good targets
for an immune response and are good malaria vaccine candidates
because merozoites are the only stage in the asexual blood
cycle in which the parasite is exposed to the immune system.
The 190kD glycoprotein of Plasmodium falciparum, precursor to
major merozoite surface antigen1 (MSA1), which is synthesized
during schizogony, is considered a promising candidate for a
blood-stage malaria vaccine (Blackman, et al., Mol. Biochem.
Parasitol., 1991, 49, 29-34; Perrin, et al., J. Exp. Med.,
1984, 160, 441-451; Siddiqui, et al., Proc. natl. Acad. Sci.
USA, 1987, 84, 3014-3018; Perrin, et al., Immunol. Rev., 1982,
61, 245-269). The high-molecular weight precursor is
processed into 88kD, 30kD, 38kD and 42kD fragments which
remain as complexes on the merozoite surface (Holder, et al.,
Parasitology, 1987, 94, 199-208; McBride and Heidrich, Mol.

<u>Biochem. Parsitol.</u>, 1987, 23, 71-84). The complex is released
from the membrane by cleavage of the 42kD anchor fragment, and
a 19kD carboxyl-terminal fragment remains on the merozoite
membrane and is carried into the invaded erythrocytes (Black-
man, et al., <u>supra</u>; Blackman, et al., <u>Mol. Biochem.
Parasitol.</u>, 1991, 49, 35-44). The complete MSA1 of
unprocessed P. falciparum has been used to provide partial or
complete protection against challenge infection (Blackman, et
al., <u>Mol. Biochem. Parasitol.</u>, 1991, 49, 29-34; Perrin, et
al., <u>J. Exp. Med.</u>, 1984, 160, 441-451; Siddiqui, et al., <u>Proc.
natl. Acad. Sci. USA</u>, 1987, 84, 3014-3018), and it is highly
immunogenic in humans (Perrin, et al., <u>Immunol. Rev.</u>, 1982,
61, 245-269). Rabbit antibody against the C-terminal process-
ing fragment of MSA1, as expressed in baculovirus, strongly
inhibits parasite growth <u>in vitro</u>. These antibodies were able
to inhibit homologous and heterologous parasites with similar
degrees of efficiency (Hui, et al., <u>Infect. Imm.</u>, 1993, 61,
3403-3411).

     In prior work at Georgetown University, a series of
monoclonal antibodies (mAbs) directed against glycophorin A,
the putative erythrocyte receptor for <u>P. falciparum</u> were
prepared. One of these mAbs, designated 2B10 is capable of
blocking the binding of MSA1 to human erythrocytes and
inhibiting the invasion of P. falciparum merozoites into human
erythrocytes (Su, et al. <u>Infect. Imm.</u>, 1993, 151, 2309). The
anti-idiotype antibody of 2B10 recognized the C-terminal
(1047-1640aa) region of MSA1 in a western blot (Su, et al., <u>J.
Immunol.</u>, 1995) and appears to recognize the same site on
glycophorin A as the merozoite.

Summary of the Invention

     The present invention relates to a malaria vaccine
comprising an expression vector, preferably, a vaccinia virus
system which expresses a protein corresponding substantially
to a specific domain of the major merozoite surface antigen 1
(MSA1) of <u>Plasmodium falciparum</u> or an immugenic peptide por-
tion thereof.

In this preferred vaccinia virus system, the DNA
coding for the MSA1 protein domain is expressed by the vac-
cinia virus after administration to a patient. The MSA1
protein or sub-fragment which is then expressed in the patient
raises a humoral and/or cell-mediated response to the
merozoite malaria antigen, which response provides the effect
of protecting the vaccinated patient from a subsequent malaria
infection. In preferred embodiments according to the present
invention, the vaccinia virus system continues to express
antigen in the patient for a period of days, months or even
years, thus reinforcing the immunogenic response of the
patient to the expressed antigen.

The MSA1 peptide antigen or immunogenic peptide por-
tion thereof which is expressed by the expression vector vac-
cinia virus may also comprise a signal peptide and/or an
anchor peptide sequence. It has been found that the addition
of a signal and/or anchor peptide to the expressed MSA1
antigen in vaccines according to the present invention unex-
pectedly enhances the immunogenicity to the patient of the
MSA1 protein of Plasmodium falciparum. It is an unexpected
result that the inclusion of a signal and/or anchor protein
with MSA1 can be expressed by a vaccinia virus system accord-
ing to the present invention and the expressed peptide will
produce a significantly greater immunogenic response than the
MSA1 peptide alone or in combination with an adjuvant. It is
also an unexpected result that the inclusion of a signal and
anchor sequence in the MSA1 peptide sequence expressed by the
vaccinia virus will produce an immunogenic response which may
be as much as 100 fold greater than the immnogenic response
which is produced by the MSA1 peptide which does not contain a
signal or anchor peptide sequence.

Methods of inducing an immunogenic response in a
patient are also contemplated by the present invention. In
this method, a patient is administered an amount of a vaccinia
virus capable of expressing the MSA1 peptide of Plasmodium
falciparum such that the patient develops an immunogenic

response to the expressed peptide. The immunogenic response generated preferably will be "substantially protective", i.e., will protect the patient from some of the more severe symptoms and physiological states of the malaria disease, including the death of the patient from malaria.

The present invention also relates to an immunogenic dosage form as a vaccine, for inducing an immunogenic response to the merozoite stage in the life cycle of Plasmodium fal-ciparum. Methods of vaccinating a patient against a malaria infection are also contemplated by the present invention. In this method, a patient is vaccinated against a Plasmodium fal-ciparum infection by administering an immunogenic response producing effective amount of a vaccinia virus capable of expressing the MSA1 peptide or an immunogenic peptide portion thereof of Plasmodium falciparum in the patient.

The present invention also relates to chimeric proteins or peptides comprising the peptide sequence cor-responding to the major merozoite surface antigen 1 (MSA1) of Plasmodium falciparum or an immugenic peptide portion thereof in combination with a signal sequence and/or anchor sequence, more preferably both a signal sequence and an anchor sequence.

Description of the Figures

Figure 1 is a diagrammatic representation of the con-struction of recombinant vaccinia viruses incorporating the sequences corresponding to MSA1C-(Si,A), MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C-(nSi,nA).

Figures 2-5 represent the gene sequences for MSA1C-(Si,A), MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C-(nSi,nA).

Figure 6 is a diagrammatic representation of four recombinant vaccinia viruses of the present invention: rv-MSA1-(Si,A); rv-MSA1-(Si,nA); rv-MSA1-(nSi,A); rv-MSA1-(nSi,nA). This figure shows the schematic of the genome of recombinant vaccinia viruses expressing the different MSA1

constructs. Tk$_1$ and TK$_R$, right and left regions ofthe vac-
cinia virsu thymidine kinase gene; LacZ, beta-galacosidase
gene. Si: signal region of MSA1; nSi: no signal region of
MSA1; A: anchor region of MSAa1; nA: no anchor region.

     Figure 7 is a diagrammatic representation of the
results obtained from Western blot analysis of recombinant
vaccinia virus expressed proteins using anti-MSA1C-A mouse
serum as a probe, of proteins expressed from BSC-1 cells
infected with vaccinia virus. A. pellet of rV.V-MSA1C(Si,nA)
infected cells; B. supernatant of rV.V-MSA1C(Si,nA) infected
cells; C. pellet of rV.V-MSA1C(Si,A) infected cells; D.
supernatant of rV.V-MSA1C(Si,A) infected cells; E. pellet of
rV.V-MSA1C(nSi,nA) infected cells; F. supernatant of rV.V-
MSA1C(nSi,nA) infected cells; G. pellet of rV.V-MSA1C(nSi,A)
infected cells; H. supernatant of of rV.V-MSA1C(nSi,A)
infected cells; I. pellet of WR infected cells; J. super-
natant of WR infected cells.

     Figure 8 is a diagrammatic representation of results
obtained from immunofluorescence staining of non-permeabilized
infected cells. HeLa cells seeded on pre-treated coverslips
for 48 hours were infected with recombinant and wild-type vac-
cinia virus at an M.O.I. of 5. The cells were probed with
anti-MSA1C-A serum and laveled with FITC goat anti-mouse.
A,B: rV.V-MSA1C(Si,nA) infected cells; C,D: rV.V-MSA1C(Si,A)
infected cells; E,F: rV.V-MSA1C(nSi,nA) infected cells; G,H:
rV.V-MSA1C(nSi,A) infected cells; I,J: WR infected cells.

     Figure 9 is a diagrammatic representation of results
obtained in rabbits immunized intradermally with the recom-
binant vaccinia viruses of the present invention. Five rab-
bits were immunized intradermally with recombinant and wild-
type vaccinia virus, and one rabbit was immunized
intravenously with rV-MSA1C-(Si,A). The I.D. immunizations
occurred on days 0, 21, 47 and 68, and the I.V. immunizations
occurred on days 0, 47 an 68. Blood samples taken on days 33,
54, and 78 were analyzed for antibodies to the trpE-MSA1C-A
protein (C-terminus of MSA1 expressed and purified from E.

coli) using ELISA. Sera were analyzed in duplicate. (+)rV-
MSA1C-(Si,A); (▲)rV-MSA1C-(Si,A);
(o)rV-MSA1C-(Si,nA); (X)rV-MSA1C-(nSi,nA); (◇)rV-MSA1C-
(nSi,A); (∫)WR.

Figure 10 is a diagrammatic representation of results
obtained in Balb/c mice which were inoculated with vaccinia
viruses of the present invention. Balb/c mice were inoculated
I.P. with recombinant and wild-type vaccinia virus on days 0,
21, 42 and 63. Blood samples taken on days 11, 31, 51, 73 93,
123, 147, 167 and 187 were analyzed for antibodies to trpE-
MSA1C-A protein (C-terminus of MSA1 expressed and purified
from E. coli) using ELISA. Sera were analyzed in duplicate:
(∫) rV-MSA1C-(Si,A); (o) (rV-MSA1C-(Si,nA); (X)(rV-MSA1C-
(nSi,A); (◇) (rV-MSA1C-(nSi,nA); (+) WR.

Figure 11 is a diagrammatic representation of the
results obtained from a Western blot analysis of the recogni-
tion of a 190kD of P. falciparum by vaccinia virus induced
mouse antibodies. Schizont stage parasites were lysed by
boiling in sample buffer and loaded onto a 4-20% Tris-glycine
gradient gel, and the proteins were transferred to a PVDF mem-
brane by electrophoresis. These blots were then probed with
anti-MSA1C-(Si,A), anti-MSA1C-A or anti-WR antibodies and
labelled with phasphatase-conjugated goat anti-mouse IgG.
A. anti-MSA1C(Si,A); B. anti-MSA1C-A; C. anti-WR.

Detailed Description of the Invention

The following terms shall be used throughout the
instant specification in an effort to describe the present
invention.

The term "chimeric protein", "chimeric peptide" or
"chimeric peptide sequence" is used to describe the non-
natural peptide sequences according to the present invention
which comprise the expressed protein or peptide and an anchor
peptide and/or a signal peptide. As noted by the use of this
term, chimeric peptides generally are synthetic peptides pro-

duced by an expression vector which contain a desired target
protein or peptide (either MSA1 or an immunogenic peptide por-
tion thereof) in combination with another peptide sequence
(either an anchor or signal peptide sequence).

The term "patient" is used to describe an animal,
including a mammal and especially including a human patient
which is administered a dosage form of an expression vector or
chimeric protein according to the present invention. In the
present invention, the expression vector encodes for MSA1 or
an immunogenic peptide portion therof and expresses the
encoded protein or peptide in the patient.

The term "expression vector" is used to describe the
means by which nucleic acid, including DNA, cDNA, RNA or
variants thereof, more preferably DNA fragments, encoding for
a specific peptide or protein, may be introduced into the
patient or the patient's tissue in order to express or produce
the desired protein. Such vectors include any vectors into
which a nucleic acid sequence encoding for the desired MSA1
protein or immunogenic peptide fragment thereof, anchor pep-
tide sequence and/or, signal protein sequence may be inserted
(along with any required or optional operational elements)
into a host organism and replicated. Expression vectors may
also be used to simply produce chimeric peptide in culture for
isolation. Preferred vectors are those which are capable of
expressing the peptide or protein sequences in mammalian cells
and whose restriction sites are well known and which contain
the required operational elements for expression of the
desired protein or peptide sequence. In the present inven-
tion, the vector is preferably a vaccinia virus vector,
adenovirus vector or herpes virus vector which has the capa-
city to infect a mammalian cell and express or synthesize
proteins utilizing the host's biosynthetic mechanism. In such
cases, the viral vector used for delivery should optimally be
one which infects cells but which does not cause lysis due to
replication (i.e., an attenuated or partially disabled virus
selected from among adenovirus, vaccinia virus and herpes
viruses, among similar types).

According to the vector approach in the present invention, the vector will infect the host cells and, using the host cells' biosynthetic pathways, synthesize encoded protein or peptide fragment. Any immunizing vehicle which has a detailed genetic and human use history may be used as the expression vector in the present invention. The preferred expression vector is a viral vector, more preferably, a vaccinia virus vector, for example, as described by Earl and Moss, Current Protocols in Molecular Biology, 1993, 16.17.1-16.17.16) and Smith and Moss, Gene, 1983, 25, 21-28. However, any vaccinia or other viral vector which may be used in the above-described manner may be appropriate for use in the present invention.

In order to express the desired protein or peptide sequence, the expression vector should contain at least one promoter, at least one operator, at least one terminator codon, and any other sequences which are necessary for the efficient transcription and subsequent translation of the nucleic acid from the vector. These operational elements are well known to those of ordinary skill in the art. In preferred embodiments according to the present invention, the expression vectors will advantageously comprise at least one origin of replication which is recognized by the host organism along with at least one selectable marker and at least one promoter sequence capable of initiating transcription of the nucleic acid (preferably, DNA) sequence.

The term "vaccine" is used throughout the specification to describe a preparation intended for active immunological prophylaxis. In the present invention, vaccines comprise an expression vector, preferably a vaccinia virus system which expresses an antigenic protein after administration of the vaccinia virus system to an animal, such as a mammal. Vaccines may also comprise chimeric peptides comprising MSA1 or an immunogenic peptide portion thereof in combination with a signal peptide sequence and/or an anchor peptide sequence. The method of administering the vaccine(s) according to the

present invention may vary and include intravenous, buccal,
oral, transdermal and nasal, among others, but intramuscular
or subcutaneous administration is the most common method of
administration.

The term "MSA1 protein" or "MSA1 peptide" is used to
describe the major merozoite surface antigen 1 of the
merozoite stage of Plasmodium falciparum or immunogenic pep-
tide portions thereof. MSA1 is the major surface protein of
the merozoite stage of Plasmodium falciparum. It is a 190kD
glycoprotein which is synthesized during schizogony life-cycle
stage. The high molecular weight precursor is processed into
88kD, 30kD, 38kD and 42kD fragments which remain as complexes
on the merozoite surfaces. The complex is released from the
merozoite membrane by cleavage of the 42kD anchor fragments
and a 19kD carboxy-terminal fragment remains on the merozoite
membrane and is carried into the invaded erythrocytes. The
complete MSA1 gene sequence is available on the data base
UNDP/WORLDBANK/WHO/TDR Malaria Sequence. The preferred por-
tion of MSA1 for expression in the vaccinia virus system
according to the present invention is a carboxy-terminal
region (corresponding to amino acids 1047 to 1640) of MSA1.
In the present invention, the expressed protein may be MSA1 or
any portion thereof, preferably such that the MSA1 portion
contains at least the carboxy-terminal region of MSA1.

The terms "carboxy-terminal region of MSA1" and
"carboxy- terminal MSA1 peptide" are used to describe that
portion of the MSA1 protein corresponding to amino acids 1047
to 1640 which is the preferred expressed antigen peptide
sequence in the present vaccines. It represents a preferred
target for the development of humoral and/or cell mediated
response because of the degree of specificity of the immune
response which can be elicited against such a protein segment.
In the present invention, the expression of the C-terminal
MSA1 peptide according to the instant invention produces a
specific immune reactivity which becomes less specific as more
of the MSA1 protein is incorporated into the vaccinia virus
system. In the present invention, the above terms to

d\escribe the C-terminal MSA1 peptide include not only the 593 amino acid peptide referred to above, but any peptide substantially corresponding to that 593 amino acid peptide.

The following DNA sequence corresponding to the C-terminus is preferably used in the present invention:

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TTGAATTC ACTTAATAAC CCAAAGCATG TATTACAAAA CTTTTCTGTT
TTCTTTAACA AAAAAAAGA AGCTGAAATA GCAGAAACTG AAAACACATT
AGAAAACACA AAAATATTAT TGAAACATTA TAAAGGACTT GTTAAATATT
ATAATGGTGA ATCATCTCCA TTAAAAACTT TAAGTGAAGA ATCAATTCAA
ACAGAAGATA ATTATGCCAG TTTAGAAAAC TTTAAAGTAT TAAGTAAATT
AGAAGGAAAA TTAAAGGATA ATTTAAATTT AGAAAAGAAA AAATTATCAT
ACTTATCAAG TGGATTACAT CATTTAATTG CTGAATTAAA AGAAGTAATA
AAAAATAAAA ATTATACAGG TAATTCTCCA AGTGAAAATA ATACGGATGT
TAACAATGCA TTAGAATCTT ACAAAAAATT TCTCCCAGAA GGAACAGATG
TTGCAACAGT TGTAAGTGAA AGTGGATCCG ACACATTAGA ACAAAGTCAA
CCAAAGAAAC CAGCATCAAC TCATGTAGGA GCAGAGTCTA ACACAATAAC
AACATCACAA AATGTCGATG ATGAAGTAGA TGACGTAATC ATAGTACCTA
TATTTGGAGA ATCCGAAGAA GATTATGATG ATTTAGGACA AGTAGTAACA
GGAGAAGCAG TAACTCCTTC CGTAATTGAT AACATACTTT CTAAAATTGA
AAATGAATAT GAGGTTTTAT ATTTAAAACC TTTAGCAGGT GTTTATAGAA
GTTTAAAAAA ACAATTAGAA AATAACGTTA TGACATTTAA TGTTAATGTT
AAGGATATTT TAAATTCACG ATTTAATAAA CGTGAAAATT TCAAAAATGT
TTTAGAATCA GATTTAATTC CATATAAAGA TTTAACATCA AGTAATTATG
TTGTCAAAGA TCCATATAAA TTTCTTAATA AAGAAAAAAG AGATAAATTC
TTAAGCAGTT ATAATTATAT TAAGGATTCA ATAGATACGC ATATAAATTT
TGCAAATGAT GTTCTTGGAT ATTATAAAT ATTATCCGAA AAATATAAAT
CAGATTTAGA TTCAATTAAA AAATATATCA ACGACAAACA AGGTGAAAAT
GAGAAATACC TTCCCTTTTT AAACAATATT GAGACCTTAT ATAAAACAGT
TAATGATAAA ATTGATTTAT TTGTAATTCA TTTAGAAGCA AAAGTTCTAA
ATTATACATA TGAGAAATCA AACGTAGAAG TTAAAATAAA AGAACTTAAT
TACTTAAAAA CAATTCAAGA CAAATTGGCA GATTTTAAAA AAAATAACAA
TTTCGTTGGA ATTGCTGATT TATCAACAGA TTATAACCAT AATAACTTAT
TGACAAAGTT CCTTAGTACA GGTATGGTTT TTGAAAATCT TGCTAAAACC
GTTTTATCTA ATTTACTTGA TGGAAACTTG CAAGGTATGT TAAACATTTC
ACAACACCAA TGCGTAAAAA AACAATGTCC ACAAAATTCT GGATGTTTCA
GACATTTAGA TGAAAGAGAA GAATGTAAAT GTTTATTAAA TTACAAACAA
GAAGGTGATA AATGTGTTGA AAATCCAAAT CCTACTTGTA ACGAAAATAA
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TGGTGGATGT GATGCAGATG CCAAATGTAC CGAAGAAGAT TCAGGTAGCA
ACGGAAAGAA AATCACATGT GAATGTACTA AACCTGATTC TTATCCACTT
TTCGATGGTA TTTTCTGCAT TTCCTCTAAC TTCTTAGGAA TATCATTCTT
ATTAATACTC ATGTTAATAT TATACAGTTT CATTTAA
```

The amino acid sequence corresponding to the above-described C-terminus of MSA1 is:

KLNSLNNPKHVLQNFSVFFNKKKEAEIAETENTLENTKILLKHYKGLVKYYNGE
SSPLKTLSEESIQTEDNYASLENFKVLSKLEGKLKDNLNLEKKKLSYLSSGLHHLIAELKEV
IKNKNYTGNSPSENNTDVNNALESYKKFLPEGTDVATVVSESGSDTLEQSQPKKPASTHVGA
ESNTITTSQNVDDEVDDVIIVPIFGESEEDYDDLGQVVTGEAVTPSVIDNILSKIENEYEVL
YLKPLAGVYRSLKKQLENNVMTFNVNVKDILNSRFNKRENFKNVLESDLIPYKDLTSSNYVV
KDPYKFLNKEKRDKFLSSYNYIKDSIDTDINFANDVLGYYKILSEKYKSDLDSIKKYINDKQ
GENEKYLPFLNNIETLYKTVNDKIDLFVIHLEAKVLNYTYEKSNVEVKIKELNYLKTIQDKL
ADFKKNNNFVGIADLSTDYNHNNLLTKFLSTGMVFENLAKTVLSNLLDGNLQGMLNISQHQC
VKKQCPQNSGCFRHLDEREECKCLLNYKQEGDKCVENPNPTCNENNGGCDADAKCTEEDSGS
NGKKITCECTKPDSYPLFDGIFCSSSNFLGISFLLILMLILYSFI.

Any expressed peptide which substantially corresponds
to the MSA1 protein or an immunogenic peptide portion of the
MSA1 protein and, preferably, also contains a peptide cor-
responding to at least an immunogenic portion of the C-
terminal MSA1 peptide, may be used in the present vaccines.
Of course, expressed peptides corresponding to MSA1 protein
and/or an immunogenic peptide portion thereof in combination
with a signal sequence or anchor sequence may also be used in
the present invention.

The term "signal peptide" "signal sequence" or "signal
protein" is used to describe a 7-30 unit amino acid peptide
sequence, preferably about a 15-26 unit amino acid peptide
sequence, which is generally found at or near the N-terminus
of the expressed protein or peptide which is used in the pres-
ent invention in order to substantially enhance the biological
activity of the protein or peptide expressed in the patient
according to the present invention. Signal sequences
generally contain hydrophobic peptide sequences of between
about 7 and 30 amino acid units, more preferably, about 15 to

26 amino acid units, even more preferably about 16 to 24 amino
acid units and most preferably about 18 to 20 amino acids
units appear to be essential for the targeting of protein
chains (generally, secretory proteins) to membranes within the
cell. These hydrophobic sequences are of sufficient length to
cross the lipid bilayer of the cell membranes. Signal
sequences serve as organizers for the cellular traffic of mac-
romolecules. These proteins are believed to play a central
role in the translocation of polypeptide chains across mem-
branes. In the present invention, the incorporation of a sig-
nal protein sequence at the amino terminus of the protein or
peptide sequence expressed by the vaccinated patient is asso-
ciated with the substantial enhancement in the biological
activity (including the therapeutic effect of immunogenicity)
associated with the expressed protein or peptide. In the
present invention, signal sequences which are known in the art
may be used in the present invention. For example, although
it may be possible to utilize yeast or lower trophic order
signal sequences, clearly mammalian signal sequences are
preferred for use in mammals and the specific species signal
sequences are most preferred for use in the desired mammalian
species to be treated. Thus, in providing for an expressed
protein or polypeptide in humans, a human signal sequence is
most preferred.

Signal sequences for use in the present invention
generally contain three regions, a first or c region at the
carboxy end of the peptide (which serves as the cleavage site
for a signal peptidase enzyyme), comprising about 5 to 7 amino
acid residues which tend to be highly polar but uncharged; a
second or h region which is N-terminal to the c region,
generally about 7 to 13 amino acid residues in length and
highly hydrophobic (comprised primarily of Leu, Ala, Met, Val,
Ile, Phe, and Trp amino acids, but may contain an occasional
Pro, Gly, Ser or Thr amino acid residue); and a third region
or n-region of highly variable length and composition, but
generally carrying a net positive charge contributed by the N-
terminus (negative charges contributed from acidic residues
are also known) and any charged residues. Between the c

region and the h region are between 1 and 3 amino acid
residues which tend to be small and uncharged (Ala, Gly, Ser,
others).   Synthetic homopolymeric h regions comprised of amino
acids selected from the group consisting of leucine,
isoleucine, phenylalanine, valine, alanine and tryptophan,
preferably leucine, isoleucine and phenylalanine may be used
in the signal proteins according to the present invention.
See generally, von Heijne, _European Journal of Biochemistry_,
(1983), 133, pp. 17-21.

        The signal sequences which are used in the present
invention preferably encompass eukaryotic signal sequences,
preferably between 7 and 30 amino acid units in length,
preferably between 15 and 26 units, more preferably between
about 16 and 26 amino acids, even more preferably between 18
and 20 amino acid units.   In the present invention, the c
region of the signal peptide should be more polar and the
boundary between the h and c regions between residues -5 and -
6, or -7 or -8 (counting from the position of cleavage of the
signal sequence- i.e., the first amino acid of the mature or
expressed protein or peptide is +1) is between 1 and 3 amino
acid residues which tend to be small and uncharged (Ala, Gly,
Ser, others).   Position preferences in the h/c for amino acids
are as follows:

        -10   most preferably leucine or alternatively,
isoleucine, valine, alanine, or phenylalanine;

        -9   most preferably leucine, alternatively,
isoleucine, alanine, valine, phenylalanine;

        -8   most preferably leucine, alternatively isoleucine,
alanine, valine, glycine, phenylalanine;

        -7   most preferably alanine, alternatively, leucine,
isoleucine, valine, phenylalanine;

        -6   most preferably valine, alternatively leucine,
valine, isoleucine, phenylalanine, alanine;

-5   most preferably proline, alternatively glycine, alanine, leucine, valine;

-4   most preferably glycine, alternatively proline, leucine, alanine, valine;

-3   most preferably alanine, alternatively valine;

-2   most preferably leucine, alternatively phenylalanine;

-1   most preferably alanine, alternatively glycine.

In the signal sequences used in the present invention, the h region may vary in length as well. The n region is polar, contains positively charged amino acids (predominantly lysine and arginine) and varies with the overall length of the signal peptide as described above. The c region extends from residues -1 to -5 of the signal peptide/expressed or mature protein. In terms of location of the c, h and n regions, the c region is N-terminus to the expressed or mature protein, the h region is N-terminus to c region (with a 1-3 amino acid boundary between the c and h region) and the n region is a positively charged N-terminus to the h region. In sum, the n region is variable in length and generally positively charged (with a preferred charge of +2), the h region is hydrophobic and variable in length and the c region preferably contains about five (5-7) generally polar amino acids.

The end of the hydrophobic domain (i.e., the boundary between the hydrophobic residues enumerated above) should preferably be at positions -6/-5. Overall, the signal sequence should comprise a 5 to 10 unit residue initial sequence (beginning with methionine) followed by at least a seven residue sequence (as described above) and an additional amino acids from 1 to 10 residues in length. A typical sequence for the region noted about is:

ILLLLAV.

The signal sequence used should be characteristic of
the cell type used for expression of the protein. Thus, in
veterinary applications, the signal sequence most preferably
used should be that of the animal to be treated. Often a sig-
nal sequence which is mammalian in character is acceptable.
Most mammalian signal sequences will have significant efficacy
in expressing proteins or peptides in other mammalian cells.
Human signal sequences are preferably used for human applica-
tions.

In the present invention, the following signal peptide
DNA sequences are preferably used:

ATG AAGATCATAT TCTTTTTATG TTCATTTCTT TTTTTTATTA TAAATACACA
ATGTG; and

ATG AAGATCATAT TCTTTTTATG TTCATTTCTT TTTTTTATTA TAAATACACA
ATGTGTAACA CATGAAAGTT ATCAAGAACT TGTCAAAAAA CTAGAAGCTT
TAGAAGATGC AGTATTGACA GGTTATAGTT TATTTCAAAA GGAAAAAATG
GTATTAAATG AA.

The amino acid sequences corresponding to the above-
described signal peptide DNA sequences are:

MKIIFFLCSFLFFIINTQC; and

MKIIFFLCSFLFFIINTQCVTHESYQELVKKLEALEDAVLTGYSLFQKEKMVLNE.

The term "anchor protein" or "anchor peptide sequence"
is used to describe proteins or peptides which are anchored to
the external surface of the plasma membrane generally by
covalent bonding to glycans containing phosphatidyl inositol.
These structures to which the anchor protein or peptide is
bonded are often referred to as glycosyl phosphatidylinositols
or GPIs. In all cells, anchor proteins covalently bonded to
GPIs are found on the external face of the plasma membrane of
cells or on the lumenal surface of secretory vesicles.

In the present invention an "anchor protein" or
"anchor peptide" comprises a peptide sequence preferably of
about 15-35 residues in length which is generally expressed at
the carboxy-terminus of the protein or peptide expressed by
the expression vector according to the present invention (3'
end of the DNA sequence expressing the desired protein or pep-
tide and carboxyl terminus of the expressed protein or pep-
tide).

In the present invention, many of the proteins or pep-
tides which are expressed in the patient and in particular,
the immunogenic proteins or peptides of vaccines according to
the present invention which are expressed in the patients pro-
duce a biological or immunogenic response in the patient which
is substantially enhanced when an anchor peptide is incor-
porated at the carboxy terminus of that protein or peptide.
The inclusion of a signal protein at or in the proximity of
the N-terminus, in addition to the anchor peptide at the
carboxy-terminus of the expressed protein, is associated with
an unexpected enhancement in the biological effects of the
expressed protein. This is especially true where the
expressed protein is antigenic or immunogenic in nature.

The carboxy-terminus of the expressed protein or pep-
tide residue is modified by attachment of a glycolipid anchor,
which serves to anchor the modified protein or peptide to the
cell surface. The peptide residue to which the GPI anchor is
added is always one of small amino acids, such as glycine,
aspartic acid, asparagine, alanine, serine and cysteine.
These occur at the carboxyl terminus of the protein/peptide of
interest and thus can be specified by inclusion of the
appropriate codons in the DNA fragment to be added to the cDNA
sequence specifying the protein/peptide of interest. In addi-
tion, the two residues downstream of the anchor addition site
are usually small.

The cleavage/anchor addition site resides in a domain
of three small amino acid residues, although the central of

the three residues has less stringent steric requirements. In
order to be certain that functionally or immunologically
important amino acids at or near the carboxyl terminus of the
protein/peptide target are not compromised, several additional
amino acids (preferably, polar ones such as lysine or arginine
as well as threonine, alanine and proline) to make up a total
of up to 10 residues are inserted in such an orientation so
that the small, polar segment is at the carboxyl terminus.
The remainder of the addition signal sequence will contain
from 15 to 35 amino acids with a hydrophobic domain at the
extreme carboxyl terminus. This domain should extend for 15-
25 amino acids and will include amino acids such as valine,
leucine, isoleucine, alanine, pphenylalanine, but may also
contain proline and glycine as well as tryptophan. A typical
such sequence is as follows:

       TACDLAPPAGTTD   AAHPGRS<u>VVPALLPLLAGTLLLLETATAP</u>

       The small sequence is in bold face with the left por-
tion represeting the terminus of the protein and the D residue
the site of GPI addition. The right hand portion is that
cleaved during GPI addition with the underlined sequence
indicating the hydrophobic terminus.

       In the present invention, the anchor peptide may have
a cleavable N-terminal sequence, which directs the peptide to
the endoplasmic reticulum and the cellular trafficking pathway
where the GPI anchor is added. As described above, the anchor
peptide also has a predominantly hydrophobic sequence at the
extreme carboxy terminus which generally ranges in size from
about 15 to about 35, more preferably about 15 to 30, and even
more preferably about 15 to 25 amino acid residues, signals
the addition of the GPI anchor and is cleaved off concurrent
with GPI addition. It is the hydrophobicity rather than the
sequence itself which is important for anchor addition.
Essentially any hydrophobic amino acid sequence of at least
about 15 to about 35, more preferably about 15 to 30 amino
acid residues would be capable of directing the addition of a
GPI anchor. Anchor addition is generally a transamidation

reaction in which the free ethanolamine amino group of the GPI
precursor attacks (by way of nucleophilic addition) a peptide
bond at the target amino acid, which becomes the C-terminal
amino acid.

Generally, in the expressed anchor peptide sequence,
just upstream of the hydrophobic sequence to which the GPI
anchor is added is a hydrophilic spacer (usually about 5-10
residues) which contains hydrophilic amino acids. The residue
to which the GPI anchor is added (the "anchor addition site")
is an amino acid residue within this hydrophilic spacer
selected from the group consisting of glycine, aspartic acid,
arginine, asparagine, alanine, serine and cysteine. In addi-
tion, the two residues downstream from the anchor addition
site are also usually small amino acid residues apparently to
minimize steric hindrance at the anchor addition site.

Preferably, the GPI portion is preassembled and added
as a single unit to a specific amino acid residue near the
carboyxl terminus of the expressed protein or peptide. Thus,
the carboxyl terminal region may be characterized by the
presence of a C-terminal signal peptide whichis preferably ten
to thirty amino acids in length and provides the information
needed to add the GPI anchor. The actual amino acid residue
to which the GPI structure is attached is called the omega
site and this residue should be glycine, alanine, cysteine,
serine, asparagine or aspartic acid. The omega +1 site
(towards the carboxyl terminus of the expressed, unprocessed
protein) preferably is selected from glycine, alanine,
cysteine, serine, asparagine, aspartic acid, glutamate and
threonine. The omega +2 site is alanine or glycine. The
omega +2 site is followed by a hinge or spacer of ideally 5 to
7 amino acids that preferably contains charged amino acids and
proline; this is followed in turn by a preferably hydrophobic
sequence of amino acids which terminate the carboxyl signal
peptide.

The overall structure of the anchor peptide may be
summarized as a 15-35 amino acid peptide at the carboxyl

terminus of the expressed protein or peptide. This anchor
peptide sequence (reading from the terminus towards the amino
end) begins with a hydrophobic stretch of amino acids of vari-
able length, followed by a sequence of preferably 5-7 amino
acids which contains charged residues, followed by three amino
acids (either glycine or alanine at the omega +2 site); any of
glycine, alanine, cysteine, serine, asparagine, aspartic acid,
glutamate and threonine at the +1 omega site; and any of
glycine, alanine, serine, cysteine, aspartic acid or
asparagine at the omega site.

        It is noted that in the present invention, while the
signal peptide sequence is generally found at the N-terminus
(directly at the N-terminus or removed as much as 1,000 or
more amino acids from the N-terminus) and the anchor peptide
sequence is generally found at the carboxy-terminus of the
expressed protein or peptide, the signal peptide may be found
at or near the carboxy terminus of the expressed target
protein or peptide.

        In the present invention, anchor sequences which are
known in the art may be used in the present invention. For
example, although it may be possible to utilize yeast or lower
trophic order anchor sequences, clearly mammalian anchor
sequences are preferred for use in mammals and the specific
species signal sequences are most preferred for use in the
desired mammalian species to be treated. Thus, in providing
for an expressed protein or polypeptide in humans, a human
anchor sequence is most preferred.

        In the present invention, the following anchor peptide
DNA sequence is preferably used:

        TTCTTAGGAA TATCATTCTT ATTAATACTC ATGTTAATAT TATCCAGTTT
        CATTTAA.

        The amino acid sequence corresponding to the above-
described anchor peptide DNA sequence is:

FLGISFLLILMLILYSFI.

In experiments which evidence the utility of the broader invention, the sequences which appear in Figures 2-5 were incorporated into vaccinia virus and were expressed in experimental animals.

It is noted that in the present invention, while the signal peptide sequence is generally found at the N-terminus (directly at the N-terminus or somewhat removed- by as much as 1,000 or more amino acids from the N-terminus) and the anchor peptide sequence is generally found at or in the proximity of the carboxy-terminus of the expressed protein or peptide, the signal peptide may be found at or near the carboxy terminus and the anchor peptide may be found at or near the N-terminus of the expressed target protein or peptide.

The term "effective amount" refers to an amount or concentration of recombinant vaccinia virus effective to produce a protective immune or therapeutic response with respect to the disease malaria. In general, an effective amount of vaccinia virus which is administered to a human patient will vary depending upon a number of factors associated with that patient, including whether the patient previously has been exposed to _Plasmodium_ _falciparum_ before. An effective amount of vaccinia virus can be determined by varying the dosage of the product and measuring the resulting cellular and humoral immune and/or therapeutic responses, prior to administration. In general terms, in humans, this amount represents approximately 10^4 to about 10^7 plaque forming units, preferably about 1×10^6 to about 5×10^6 plaque-forming units (determined by assay, as described herein). It is noted that the above described range of administered vaccinia virus is chosen to enhance the likelihood of eliciting an immunogenic response without causing a malaria infection in the vaccine recipient.

In the case where the chimeric peptide is administered instead of the expression vector in order to facilitate an immunogenic response, the amount of chimeric peptide

administered will be an amount or concentration of the peptide
to produce a protective immune or therapeutic response with
respect to the disease malaria. While this amount may vary
over a considerably wide range, depending upon the
immunogenicity of the chimeric peptide chosen, generally the
amount of peptide administered ranges from about 0.01 micro-
grams (10 nanograms) to about 250 micrograms, more preferably
about 0.1 microgram to about 100 micrograms, even more
preferably about 1 microgram to about 25 micrograms within
this range.

The present invention contemplates, as a preferred
embodiment, the incorporation of both a signal peptide and
anchor peptide along with the MSA1 peptide into the expressed
peptide has been found to be particularly advantageous in
producing an immunogenic response to the MSA1 peptide. The
signal peptide sequence is generally incorporated into the
immunogenic peptide at or near the amino end of the MSA1 pep-
tide or related antigenic peptide and the anchor peptide is
generally incorporated at or near the carboxyl end of the MSA1
peptide. The immunogenic peptide is expressed by the vaccinia
virus accordingly and will contain a signal peptide aequence
and/or an anchor peptide sequence. Thus, in the present
invention, the signal and anchor peptides are preferably
expressed at the amino and carboxy terminus of the expressed
MSA1 peptide, respectively. Generally, the signal peptide
sequence is located upstream from the MSA1 peptide and the
anchor peptide is downstream from the MSA1 peptide.

In the method of preparing vaccinia virus which leads
to expression of MSA1 peptide, preferably MSA1 peptide con-
taining a signal and/or anchor peptide, by the vaccine
recipient, any method which is capable of incorporating a
sequence of DNA containing genetic material for the expression
of the MSA1 peptide and optionally, a signal peptide and/or an
anchor peptide, may be used. The method which is used in the
present invention is well known in the art. Accordingly, in
the present invention, a DNA sequence containing the genetic
code for the MSA1 peptide to be expressed is obtained by

chemical synthesis or other means such as biochemical isola-
tion of available MSA1 DNA sequences and incorporated into a
cloning plasmid (for example following cloning vectors:
pBR322, pGEM3z, pSP70, pSE420, pRSET, lambdaZAP, all commer-
cially available, among numerous others). The appropriate DNA
sequence is cloned, isolated, for example, using agarose gel
electrophoresis and then incorporated into an amplification
vector and amplified by a standard polymerase chain reaction
technique for a sufficient number of cycles to obtain a
desired quantity of DNA (depending upon the amount of DNA
desired, from about 5 cycles to about 40 cycles or more). A
signal peptide sequence and/or anchor peptide sequence may be
incorporated into a vector containing the MSA1 peptide and,
after identification (selection and screening) of the
appropriate DNA fragments in positive clones by PCR and
endonuclease digestion, amplified accordingly using the same
techniques.

        After amplification, the DNA is incorporated into a
transfer vector and transfected with eukaryotic cells, for
example, monkey kidney cells (BSC-1 cells), and with wild-type
vaccinia virus (WR) to produce recombinant vaccinia virus.
The recombinant vaccinia virus is then purified before
amplification. After amplification and in some cases further
purification, the recombinant vaccinia virus is then
administered to an animal as an immunogenic dosage form which
expresses MSA1 peptide or an immunogenic portion thereof,
preferably, in combination with a signal peptide and/or an
anchor peptide.

        Alternatively, once a nucleic acid sequence encoding
immunogenic chimeric protein is present in a suitable expres-
sion vector, the expression vector may be used for the purpose
of expressing the immunogenic chimeric protein in a suitable
eukaryotic cell system, for example, to promote the production
of the desired peptide sequence outside of the host animal.
Such eukaryotic cell systems include, for example, HeLa, L929,
T2 or RMA-2, preferably T2 or RMA-S. In this method, the
cells which contain the expression vector(s) are grown and

then lysed in order to isolate synthetic peptides which con-
tain the desired protein or peptide sequence in combination
with the anchor peptide sequence and/or the signal sequence.
The isolated peptide sequence may then be used directly as a
therapeutic or immunogenic dosage form. Alternatively and
preferably, the expression vector may be administered directly
to the patient where it will express the desired protein or
peptide and anchor sequence and render the intended
therapeutic or immunogenic effect on the patient.

The expressed protein may be obtained from cell cul-
ture after the cells are lysed by standard protein purifica-
tion procedures known in the art which may include, among
others, gel electrophesis, affinity and immunoaffinity
chromatography, differential precipitation, molecular sieve
chromatography, isoelectric focusing and ion-exchange
chromatography. In the case of immunoaffinity chromatography,
the protein or peptide may be purified by passage through a
column containing a resin to which is bound antibodies which
are specific for at least a portion of the protein or peptide.

The expressed protein or peptide containing a signal
peptide sequence and/or an anchor peptide sequence, which is
obtained from cell culture, may be administered in pure or
substantially pure form to a patient in need of such therapy
by purifying the crude lysate from cell culture. Preferably,
the expressed protein is administered in pharmaceutical dosage
form as a composition or formulation comprising an immunogeni-
cally effective amount of the expressed protein containing
anchor peptide sequence and/or signal peptide sequence, in
combination with a pharmaceutically acceptable additive, car-
rier or excipient. The formulations may be delivered in unit
dosage form prepared by known methods in the art. The amount
of expressed protein or peptide administered will vary depend-
ing upon the pharmokinetic parameters, severity of the disease
treated or immunogenic response desired. Of course, dosages
will be set by the prescribing physician considering relevant
factors including the age, weight and condition of the patient
including, in the case of immunogenic dosage forms, whether

the patient has been previously exposed to the microorganism
responsible for the disease to be vaccinated against as well
as the release characteristics of the expressed protein from
pharmaceutical dosage forms of the present invention.

The amount of the expressed protein which is
administered according to the present invention comprises an
amount effective to produce the intended effect, i.e., to
obtain an immunogenic response in the patient which provides a
substantially protective effect against malaria.

Alternatively and preferably, the vaccine which is
administered according to the present invention comprises an
amount of an expression vector, preferably, a recombinant vac-
cinia virus effective to express sufficient MSA1 peptide to
provide an immunogenic response in a patient. Preferably, the
MSA1 peptide or an immunogenic peptide sequence thereof is
combined with a signal and/or anchor peptide to substantially
increase the immunogenicity of the expressed MSA1 peptide com-
pared to MSA1 peptide which does not contain a signal and/or
anchor peptide. The immunogenic response provides a pro-
tective effect against the merozoite stage of malaria.

The present vaccine can be injected as is, or for con-
venience of administration, can be added to a pharmaceutically
acceptable carrier. Suitable pharmaceutically acceptable car-
riers will be apparent to those skilled in the art, and
include water and other polar substances, including lower
molecular weight alkanols, polyalkanols such as ethylene
glycol, polyethylene glycol, and propylene glycol as well as
non-polar carriers.

Dosages of recombinant vaccinia virus or chimeric
protein or peptide according to the present invention which
are coadministered with carriers will often be about the same
as the amount administered alone (in the absence of coad-
ministration). Of course, dosages will be set by the pres-
cribing physician considering relevant factors including the
age, weight and condition of the patient including whether the

patient has been previously exposed to <u>Plasmodium</u> <u>falciparum</u>
and the release characteristics of the vaccinia virus from
pharmaceutical dosage forms of the present invention.

In the malaria vaccine aspect of the present inven-
tion, the dose of vaccinia virus will depend upon the form in
which it is administered. For example, the vaccine will
generally contain a concentration of virus ranging from about
10^4 to about 10^7 plaque forming units, preferably about 1 X
10^6 to about 5 X 10^6 plaque-forming units, depending upon the
desired levels of expressed immunogenic protein. Thus, the
concentration or amount of vaccinia virus included within the
present vaccine will generally fall within this range;
however, the amount of recombinant vaccinia virus used in any
vaccine form will depend upon the strength of the immunogenic
response elicited.

In determining the amount of vaccinia virus in a given
vaccine dose, the following method may be used. In certain
vaccine dosage forms, standard pharmaceutical carriers as
described above may be included. The ratio of virus included
in the vaccine will depend on the chemical nature, solubility,
and stability of the virus, as well as the dosage con-
templated. For parenteral administration or injection via
such parenteral routes as intraperitoneal, intramuscular, sub-
cutaneous, intramammary or other route, sterile solutions of
the vaccinia virus are prepared. Vaccines according to the
present invention may also be administered intravenously.
Preferably, the vaccines according to the present invention
are administered via a subcutaneous route.

The dosage of the vaccine employed and the treatment
schedule would follow practices normally employed for other
vaccination or therapeutic regimens wherein this general
method of treatment is employed. It is not anticipated that
more than one dose of vaccine initially would be required, but
the possibility of providing booster doses is anticipated.
Preferably, the dosage schedule for immunization against
malaria involves the subcutaneous injection of at least about

1 X 10^6 plaque-forming units of vaccinia virus.

In the immunogenic method according to the present
invention, a human patient is administered with an effective
amount of vaccinia virus such that expressing the MSA1 peptide
or an immunogenic peptide thereof, preferably in combination
with a signal and/or anchor peptide. Alternatively, an
immunologically effective chimeric peptide comprising the MSA1
peptide or an immunogenic portion thereof in combination with
a signal peptide and/or anchor peptide will be admnistered.
In certain instances, an additional boost of vaccinia virus or
peptide may be given to promote the immunogenic response.
Additional doses of vaccine may be provided to boost the ini-
tial inoculation, if needed.

The following examples are provided for purposes of
illustration only and are not to be viewed as a limitation of
the scope of the invention.

EXAMPLES

Methods and Materials

Virus and cells

Monolayer cultures of monkey kidney cells (BSC-1) and
Hu134TK-cells were grown in Dulbecco's modified Eagle's medium
(D-MEM) supplemented with 10% fetal bovine serum (FBS).
Monolayer cultures of Hela cells and CV- cells were grown in
Dulbecco's modified Eagle's medium (D-MEM) supplemented with
10% FBS.

Wild type vaccinia virus Western Reserve (WR) was
grown in BSC-1 cells.

In vitro culture of P. falciparum

P. falciparum isolates (FCR-3, a strain identical to
the Wellcome strain sequenced by Holder, et al., Nature, 1985,

317, 270-273) were maintained in human erythrocytes in RPMI
1640 medium supplemented with 25 mM Hepes, 32mM sodium bicar-
bonate and 10% human serum in a 5% O_2, 5% CO_2, 90% N_2 environ-
ment at 37°C. P. falciparum strain FCR-3 was kindly donated by
Dr. Isabella Quakyi, Georgetown University, Washington, D.C.

Preparation of antisera to C-terminal of MSA1

pME-2 is an expression plasmid derived from pWRL507,
in which the C-terminal of the MSA1 gene (3555-5917bp,
Wellcome Allele of MSA1) was inserted at the 3' end of a trun-
cated trpE gene, so that expression was controlled by the trp
promoter. The protein was expressed using the method
described by Holder, et al., Paraite Immunol., 1988, 10, 607.
The fusion protein expressed was insoluble. DH5alpha competent
cells (GIBCO BRL) were transformed by pME-2, and a 1ml culture
of a pME-2 positive clone was innoculated into 100ml M9 medium
containing 100 ug/ml (micrograms per ml) Amp and grown over-
night. This overnight culture was added to 400ml M9 containing
100 ug/ml Amp and 10 ug/ml indoleacrylic acid, and after 5 hr.
growth at 37°C and 250 rpm, the cells were harvested. The pel-
let was then rinsed in 10 ml PBS and frozen to -20°C. The cell
pellet was thawed in a 10ml solution contiaining 25 mM Tris pH
8.0, 1mM EDTA, 0.2% NP-40 and 100 ul 100mM PMSF.

When the pellet was fully resuspended, lysozyme was
added to a final concentration of 1mg/ml, and the solution was
placed on ice for 2 hrs. After this time, 20 ul 1M MgSO4, and
20ul 10 mg/ml DNase were added, and the solution was again
left to incubate on ice for 2 hrs. After 10 min. centrifuga-
tion at 13000 rpm, the pellet was rinsed in 10 ml of washing
buffer (50 mM Tris, 5 mM EDTA, 5 mM EDGA, 1% NP-40 containing
100 ul 100 mM pMSF). The material was centrifuged as above to
yield a futher pellet and this pellet was resuspended in a 10
ml solution of 0.5M KSCN, 50mM tris, 5mM EDTA and 5mM EGTA and
re-centrifuged as above. Finally, the pellet was resuspended
in 3 ml water. The product was anayzed by SDS-polyacrylamide
gel electrophoresis (SDS-PAGE), and the gel containing the 105
kD protein of interest was then cut into approximately 1-5 mm^2

pieces. The fusion protein was eluted with a Bio-Rad Model 422 Electro-Eluter, and the vacuum-dried product was dissolved in PBS. The concentration was assayed with a BCA protein Assay Reagent Kit (PIERCE). Antiserum to the fusion protein was prepared by immunizing Balb/c mice intraperitoneally on two occasions 21 days apart with 100 ug of protein in the presence of Titre-Max (Vaxcel, Inc.). Antiserum was collected 10 days after the second immunization.

Plasmid Construction

(1) Amplification of MSA1 signal sequence (Si)

pGEX-2T-P190CR1 (pGEX-2T containing the MSA1 gene from 1 to 150bp) was used as a sample in PCR. The 100ul mixture contained 100 pmol primer 1 and 100 pmol primer 2 (Table 1, below), 2.5units Ampli Taq DNA polymerase, dNTP and 10ul 10x reaction buffer (PIERCE). The sample was overlaid with several drops of mineral oil to prevent evaporation and sub-jected to 30 cycles of amplification (94°C melt, 72°C exten-sion, 55°C annealling). Amplified products were identified on a 1.5% agarose gel.

Table 1
Relative Positions of Amplified Gene Fragments From PF MSA1 and Primer Sequences

Amplied Gene Fragments	Relative Position in MSA1 Gene[a]	Primer	Primer Sequence
Fragment Containing Signal Sequence	418-582	1	GC GTCGAC ATG AAG ATC ATA TTC TTT TTA SaII
		2	GC GAATTCAA TTC ATT TAA TAC CAT TTT TTC EcoRI
MSA1C-A	3556-5337	3	GC GAATTC ACT TAA TAA CCC AAA GCA TGT EcoRI
		4	GC GGTACC TTA AAT GAA ACT GTA TAA TAT KpnI
MSA1C-Si,nA	418-582 3553-5280	1	GC GTCGAC ATG AAG ATC ATA TTC TTT TTA SaII
		5	GC GGTACC TTA GTT AGA GGA ACT GAC GAA AAT KpnI
MSA1C-nSi,nA	475-582 3553-5280	6	GC GTCGAC ATG GTA ACA CAT GAA AGT TAT CAA SaII
		5	GC GGTACC TTA GTT AGA GGA ACT GAC GAA AAT KpnI
MSA1C,nSi,A	475-582 3553-5337	6	GC GTCGAC ATG GTA ACA CAT GAA AGT TAT CAA SaII
		4	GC GGTACC TTA AAT GAA ACT GTA TAA TAT KpnI

[a]GCG, Accession no. X02919. The start codon of MSA1 is at 418.

The MSA1C-(Si,nA), MSA1C-(nSi,nA), MSA1C-(nSi,A) fragments contain the 108bp region directly downstream from the signal sequence and an additional 2bp on the 5' end of the C-terminal to preserve the reading frame. Furthermore, a start codon was also added to the two fragments lacking the start-codon-containing signal sequence, a stop codon was also added to the two fragments lacking the anchor region.

(2) Amplification of C-Terminal of MSA1(MSA1C-A)

pME-2 plasmid was used as a template in PCR. The 100ul (microliter) mixture contained primers 3 and 4 (Tab. 1) and was subjected to 30 cycles of amplification (94°C melt, 72°C

extension, 50°C annealling). The amplified products were
identified on a 0.8% agarose gel.

pSPORT1-MSA1C-(Si,A)(Figure 1) was used as a template
in PCR. MSA1C-(Si,nA) (primer 1 and primer 5, Tab. 1), MSA1C-
(nSi,A) (primer 5 and primer 4), and MSA1C-(nSi,nA) (primer 6
and primer 5) fragments were amplified as well with the above
procedures.

(3) Isolation, purification and cloning of gene fragments

The MSA1C-A fragment was amplified by PCR, and elec-
trophoresis in a 0.8% agarose gel indicated that the amplified
fragment was about 1.8kb. This fragment and pSPORT1 were
digested with EcoRI and KpnI and were mixed and treated with
T4 DNA ligase. The ligation products were transformed into
DH5alpha competent cells. X-gal and ampicillin were used to
screen the positive clones (pSPORT1-MSA1C-A, Figure 1). Recom-
binant plasmids were prepared from positive white colonies and
identification was performed with SalI and EcoRI digestion.
The full sequence of the insert was determined.

The fragment containing the signal sequence was
amplified by PCR, and electrophoresis in a 1.5% agarose gel
indicated that the size of the amplified fragment was about
180bp. The Si and pSPORT1-MSA1C-A fragments were then digested
with SalI and EcoRI, ligated and transformed, and 30 colonies
were selected and screened by PCR using primer 1 and primer 2.
Recombinant plasmids (pSPORT1-MSA1C-(Si,A), Figure 1) were

identified with SalI and EcoRI digestion and were sequenced, and the correct reading frame was established. pSPORT1-MSA1C-(Si,A) was then used as a template to amplify MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C-(nSi,nA).

MSA1C-(Si,nA), MSA1C-(nSi,A), MSA1C-(nSi,nA) digested with SalI and EcoRI and MSA1C-(Si,A) cut from pSPORT1-MSA1C-(Si,A) were inserted separately into the SalI and KpnI sites of pSC65. Positive clones were screened by PCR and SalI and KpnI digestion, the 5' and 3' ends of each insert were sequenced, and the correct reading frame was established.

Figure 1 diagrams the construction of recombinant vaccinia viruses incorporating the sequences corresponding to MSA1C-(Si,A), MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C-(nSi,nA). Essentially, the MSA1C-terminal fragment containing the anchor region was inserted into the pSPORT1 plasmid using the marked EcoRI and KpnI sites, creating the pSPORT1-MSA1C-A plasmid. The fragment containing the signal region (FSi) and the 108 bp downstream was PCR amplified with a SALIU site on the 5' end and then inserted into the pSPORT1-MSA1C-A plasmid to produce pSPORT1-,SA1C-(Si,A) plasmid. The entire MSA1C-(Si,A) fragment (from the SalI site to the KpnI site) was then removed and inserted into the pSC65 vector to make the final pSC65-MSA1C-(Si,A) transfer vector. The other three recombinant transfer vectors (pSC65-MSA1C-(nSi,nA), pSC65-MSA1C-(nSi,A) and pSC65-MSA1C-(Si,nA)) were produced by adding the desired SalI or KpnI sites where necessary by PCR amplification. The amplified fragments were then inserted into the pSC65 plasmid.

In all four cases, the insertion site is adjacent to the
synthetic early/late promoter (pS-E/L). Tk_L and TK_R, right
and left regions of the vaccinia virus thymidine kinase gene;
LacZ, beta-galactosidase gene.

DNA sequencing

DNA sequencing was performed using the dideoxy
nucleotide chain termination method according to the protocols
for DNA sequencing with TAQ version 2.0 DNA polymerase (United
States Biochem). Figures 2-5 are the DNA (gene) sequences for
MSA1C -(Si,A), MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C-
(nSi,nA).

Transfection and isolation of recombinant vaccinia viruses

Monolayers of BSC-1 cells were grown to 90% confluence
in a six-well plate, media was removed, and the cells were
infected with wild-type vaccinia virus (WR) at 0.1-1 plaque
forming units(pfu)/cell for 1 hour. The virus inoculum was
removed, and the monolayer was washed twice with OptiMEM
(GIBCO BRL) serum-free medium. 1ml of Opti-MEM was added to
the infected monolayers and mixed gently with 50 ul of
lipofectin-DNA complex (5 ug of recombinant pSC65 was diluted
to 25 ul with sterile distilled water, 15 ug of lipofectin
reagent was diluted to 25 ul with sterile distilled water, and
the solution was gently mixed in a polystyrene tube and
allowed to stand for 15 minutes at room temperature). After 5
hours incubation at 37°C, the medium was replaced with 3 ml E-
MEM supplemented with 2% FBS and incubated for another 48
hours. After removal of the medium, cells were harvested by
scraping into 1 ml of E-MEM supplemented with 2% FBS. The
virus was released by three cycles of freeze-thawing at 37°C.
After removal of medium, 1ml of diluted freeze-thawed trans-

fection mixture (sonication 30 seconds at 4°C before adding) was added to monolayers of Hu 134 TK- grown to 90% confluence in a six-well plate, and the virus was left for 1 hour at 37°C. The infected cells were overlaid with E-MEM supplemented with 2% FBS containing 1% low-melting-point agarose and 25 ug/ml of bromodeoxyuridine. Thirty-six hours post-infection, monolayers were overlaid with E-MEM supplemented with 2% FBS containing 1% low-melting-point agarose, 0.02% neutral red and 300 ug/ml X-gal. After the agarose had set, monolayers were incubated for 6-8 hours before plaques were stained and picked (using a sterile glass Pasteur pipette) into 1 ml of diluent and freeze-thawed 3 times. The recombinant products were pla-que purified before amplification to produce small virus stocks.

Immunostaining of vaccinia recombinant plaques

        BSC-1 cells were infected with recombinant vaccinia virus, and the medium was removed from infected tissue-culture plates 24 hours postinfection. The cells were fixed with a 1:1 acetone:methanol mixture for 2 minutes, the wells were washed with 1ml of PBS, and then anti-MSA1C-A serum diluted 1:200 in PBS containing 2% FBS was added to the wells, 1 ml/well. The six-well plate was incubated at room temperature for 1 hour, rocking gently, after which the wells were washed twice with 1 ml of PBS. Anti-mouse-peroxidase diluted 1:1000 in PBS with 2% FBS was added to each well, and the plate was incubated for 45 minutes at room temperature. After washing twice with PBS, 0.5 ml of substrate solution was added (the substrate solution

was made by dissolving a pinch of dianisidine in 500ul of
absolute ethanol, vortexing, and warming it for 5-10 minutes,
then centrifuging it for 30 seconds and adding 200ul of sub-
strate solution to 10 ml of PBS plus 10ul of 30% H_2O_2). The
plate was then left for 5-10 minutes at room temperature.

Indirect immunoflourescence staining of recombinant vaccinia-infected cells

HeLa cells were seeded on pre-treated coverslips for
48h, after which the cells were infected at an M.O.I. of 5 in
a volume of 0.25 ml of D-MEM uith 10% FBS. These were over-
laid with 1.5 ml D-MEM with 10% FBS 1-2 h postinfection. The
cells were then washed with PBS and fixed for 15 min in 3%
paraformaldehyde in PBS. After being washed in PBS, the
coverslips were incubated in 50mM ammonium chloride in PBS for
10 min at room temperature. After the slips were again washed
in PBS, anti-MSA1C-A serum diluted 1:800 in PBS was added to
each well, and they were incubated for 30 min at 4°C. The
coverslips were then washed three times in PBS, and the cells
were incubated with FITC goat-anti-mouse for 30 min at 4°C.
After being washed in PBS, the coverslips were blotted on tis-
sue and mounted on slides in 5% DABCO/Mowiol.

Western blot analysis

Confluent six-well plates of BSC-1 cells were infected
with recombinant vaccinia virus in 1 ml medium at an M.O.I of
5. Cells from each well were harvested 24 hours post-
infection. After centrifugation at 8000 rpm for 5 min, the

supernatant was concentrated to 10-15 ul with a microcon 30 (Amicon, Inc.), and the cell pellet was resuspended in PBS to a final volume of 200 ul. The same volume of 2x sample buffer (100 mMTris, 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) was added, and the resulting solution was boiled for 5 min and loaded in a 8% Tris-glycine-SDS gel. Electrophoresis was performed in Tris-glycine-SDS running buffer, and proteins were transfered to a PVDF membrane by electrophoresis in transfer buffer (25mM Tris-HCl, 192mM glycine, 155 methanol) at 100V for 30 min at 4°C. The blot was blocked with 5% BSA in Tris-HCl (200mM, 0.85%) for 2 h at room temperature before addition of a 1:500 dilution of anti-MSA1C-A serum. After 2 hours incubation the blot was washed three times in Tris-HCl, pH 7.4 for 10 min per wash. A 1:7500 dilution in Tris-HCl, pH 7.4 of alkaline phosphatase-conjugated goat-anti-mouse IgG (Promega) was added, and the blot was incubated for 90 min at room temperature before being washed four times in Tris-HCl, pH 7.4 for 10 min each wash. Western blue stabilized substrate for alkaline phosphatase (Promega) was added, and after about 5 minutes, the blot was washed with water to stop development.

Large-scale preparation and plaque titration of vaccinia virus stocks

(1) purification of vaccinia viruses

$5x10^7-10^8$ HeLa S3 cells in E-MEM supplemented with 2% FBS were incubated in a 162 cm^2 flask at 37°C for 18 h, after which the cells were infected with small stock viruses at 37°C

for 48 h and pelleted by centrifugation for 5 min at 1800 g. The infected cells were resuspended in 10 mM Tris-HCl, pH 9.0 and homogenized with 30-40 strokes (while still on ice). The mixture was centrifuged for 5 min at 300g to remove nuclei, and the pellet (removed supernatant was kept was well) was resuspended in 10 mM Tris-HCl, pH 9.0 and centrifuged again (supernatants were pooled). The sonicated supernatant was layered onto a cushion of 36% sucrose in a Beckman SW27 centrifuge tube and spun at 35000 rpm for 45 min at 4°C, after which the virus pellet was resuspended in 1 ml of 1mM Tris-HCl, pH 9.0. The wild-type virus was further purified by continuous sucrose gradient centrifugation.

(2) Plaque titration of vaccinia virus stocks

Monolayers of BSA-1 cells were grown to 95% confluence in a six-well plate, virus stocks were sonicated, and 10 fold serial dilutions(10^{-7}-10^{-9}) of the virus in E-MEM supplemented with 2% FBS were made. The BSC-1 cells were infected with 1 ml of virus diluted to $10^{-7}, 10^{-8}$ and 10^{-9} in duplicate. After 48 h of incubation, the medium was removed and 0.5 ml of crystal violet solution was added. After another 15 min incubation at room temperature, the plates were washed with water, and the plaques were counted after drying.

ELISA determination of antibody response

(1) Antibody responses to MSA1C-A

A ninety-six-well microtiter plate (IMMULON 2,
Dynatech Laboratories Inc, VA) was coated with 100 ul/well of
a 5 ul/ml solution of MSA1C-A in Tris-NaCl (0.02,0.85%), pH
7.4 and kept overnight at 4°C. The antigen-coated plate was
then washed with Tris-NaCl and blocked with 150 ul 1% BSA in
Tris-NaCl, pH 7.4 for 2 h at 37°C. After washing with Tris-
NaCl, pH 7.4, 100 ul of serial 10-fold dilutions (diluted in
Tris-NaCl containing 1% BSA and 0.05% Tween 20) of sera were
added to the wells in duplicate. After 2 h incubation at 37°C,
the plate was rewashed with Tris-NaCl containing 0.05% Tween
20 and phosphatase-labeled IgG was added. After another 2 h
incubation at 37°C, the plate was washed with Tris-NaCl con-
taining 0.05% Tween 20 and developed by adding alkaline
phosphatase substrate in diethanolamine buffer, pH 9.8. The
plate was scanned in a Dynatch ELISA scanner at 405 nm.

(2) Antibody responses to vaccinia virus

Purified WR vaccinia virus was diluted in Tris-NaCl,
pH 7.4 to a final concentration of approximately 5×10^6 pfu/ml
and 100 ul volumes were dispensed into the wells of a 96-well
plate (IMMULON 2, Dynatech Laboratories Inc) and left for 2 h
at 37°C. The buffer was removed and virus was inactivated with
50 ul of 10% paraformaldehyde for 10 min at 4°C. The plates
were then blocked just as those in the first group and
developed using the procedure described above.

Indirect fluorescent-antibody tests for parasite

P. falciparum parasites were cultured in human
erythrocytes to a parasitemia of 8% at the schizont stage, at
which point they were washed five times in PBS (10x volume).
Small samples were then taken, smeared on a glass slide and
fixed in cold methanol. After being washed in PBS, the samples
were probed with antiserum from mice immunized with the recom-
binant and wild-type viruses. After this probe, the samples
were probed with FITC goat-anti-mouse antibody and mounted in
5% DABCO/ Mowiol.

In vitro invasion assay

Preparation of samples: Rabbit pre-immune serum or
anti-serum (6.7ml) was heat-inactivated at 56°C for 25
minutes, and antibodies in the absorbed serum were
precipitated with ammonium sulfate. After centrifugation, the
pellets were dissolved in a minimal volume of PBS, then
dialyzed overnight three times in PBS. Finally, the volume was
adjusted to 1 ml.

P. falciparum parasites were synchronized twice with
5% sorbitol (Diana), and after 24 hours a mixture of
trophozoites and schizonts at 3% parasitemia and 3% hematocrit
was transfered to 96-well culture plates (170ul/well). Anti-
serum, pre-immune serum or serum-free medium was added to the
parasites in a volume of 30 ul to make a final volume of
200ul/well, and the plates were incubated at 37°C in an air-
tight chamber equilibrated for 5 min with 5% O_2, 5% CO_2 and
90% N_2. After 22-24 hours, cell morphology was verified to

make sure that all schizonts had burst. Once this was verified, 4ul of homogenized infected red blood cells from each well were fixed in 2 ml 0.01% (w/v) glutaraldehyde for 45 min at room temperature. After fixing, the cells were centrifuged at 1500 rpm at 4°C for 10 min and then stained with 50ug/ml propidium iodide overnight at 4°C in darkness. Total parasitemia was determined by counting fluorescent cells using a fluorescence-activated cell sorter. 20000 cells were counted from each sample. The percentage inhibition of invasion during the 22-24 hour period was determined by the following formula:

$$\% \text{ inhibition} = \frac{(pre - 0\ hr) - (T - 0\ hr)}{(pre - 0\ hr)} \times 100\%$$

Here, "T" is the parasitemia of tested serum, "pre" is the parasitemia of preimmune serum and "0 hr" is the starting parasitemia.

Results

Construction of recombinant vaccinia virus

The construction of the recombinant vaccinia virus is
described in Figure 1. Four recombinant vaccinia virus trans-
fer plasmids were made: pSC65-MSA1C-(Si,A), which is recom-
binant pSC65 in which the MSA1 peptide containing the signal
and anchor regions of MSA1 has been inserted into the SalI and
KpnI site; pSC65-MSA1C-(Si,nA) (the same except the MSA1 con-
tains the signal without the anchor); pSC65-MSA1C-(nSi,A) (the
same except the MSA1 contains the anchor without the signal)
and pSC65-MSA1C-(nSi,nA) (the same except the MSA1 contains
neither the signal nor the anchor).

The vaccinia virus transfer vector, pSC65, has a
synthetic compound early/late promotor (pSE/L) so that the
foreign genes controlled by the promotor are expressed
throughout the virus growth cycle. The SalI, BglII, StuI,
AatI, KpnI, SmaI, XmaI and PacI sites are located just
downstream of the pSE/L for insertion of a foreign gene, and
there are E. coli beta-galactosidase gene sequences (con-
trolled by the p7.5 vaccinia virus promotor which has early
and late vaccinia virus transcriptional regulatory signals)
and vaccinia TK gene sequences flanking the entire pSE/ L and
foreign gene region to direct homologous recombinantion into
the TK locus of the vaccinia virus genome.

BSC-1 cells were infected with wild-type (WR) vaccinia

virus and then were transfected with recombinant pSC65. Serial dilutions of progeny virus were then applied to monolayers of Hu 134 TK- cells in the presence of BrdU to select TK- recombinant virus plaques. These were then distinguished from spontaneous TK- mutants by addition of X-gal to the low-melting-point agar overlay. Plaques that stained blue due to expression of beta-galactosidase were picked and then plaque-purified a second time prior to preparation of virus stocks. The four recombinant vaccinia viruses were named as rV.V-MSA1C(Si,A); rV.V-MSA1C-(Si,nA); rV.V-MSA1C-(nSi,A) and rV.V-MSA1C(nSi,nA) (Figure 6).

Expression of MSA1C-(Si,A), MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C-(nSi,nA)

(1) Immunostaining of vaccinia recombinant plaques

BSC-1 cells were infected with rV.V-N,ISA1C-(Si,A), rV.V-MSA1C(Si,nA), rV.V-MSA1C-(nSi,A) and rV.V-MSA1C-(nSi,nA), and the infected cells were fixed with acetone/methanol 24 hours post-infection, after which the expressed proteins were labeled by anti-mouse-peroxidase. The results indicated that the cells infected by the recombinant vaccinia virus expressed the C-terminal protein.

(2) Western blot analysis of recombinant vaccinia virus expressed proteins

BSC-1 cells were infected with rV.V-MSA1C-(Si,A),

rV.V-MSA1C(Si,nA), rV.V-MSA1C-(nSi,A), rV.V-MSA1C-(nSi,nA) and
WR viruses. These cells were then harvested 24 hours post-
infection, and the cell pellets and 50-times concentrated
supernatants were run on an 8% Tris-glycine-SDS gel. The blot
was labled with anti-MSA1C-A mouse serum and then probed by
alkaline phosphatase-conjugated goat anti-mouse IgG. The
results indicated that the infected cells expressed the C-
terminal region of MSA1, and that the molecular weights of
MSA1C-(Si,nA), MSA1C-(nSi,A) and MSA1C(nSi,nA) were about
70kD, and MSA1C-(Si,A) was about 60kD. Furthermore, none of
the four expressed proteins appeared to be secreted by the
cells. Figure 7 shows a Western blot analysis, using the
anti-MSA1C-A mouse serum as a probe, of proteins expressed
from BSC-1 cells infected with vaccinia virus.

       (3) Indirect immunofluorescence

       Immunofluorescence microscopy of cells on coverslips
demonstrated that MSA1C-(Si,A) and MSA1C-(Si,nA) were
expressed on the surface of infected cells, and that MSA1C-
(nSi,A) and MSA1C-(nSi,nA) were not expressed on the surface
of infected cells. Therefore, we have concluded that the sig-
nal region is vital for the expression of the protein on the
cell surface. Figure 8 shows indirect immunofluorescence
staining of recombinant vaccinia-infected cells.

       (4) Antibody response in rabbits

       Five rabbits were inoculated intradermally with rV.V-

MSA1C-(Si,A); rV.V-MSA1C-(Si,nA); rV.V-MSA1C-(nSi,nA); rV.V-
MSA1C-(nSi,A) and WR on days 0, 21, 47 and 68. The ELISA
titers of rV.V-MSA1C-(Si,A) and rV.VMSA1C-(Si,nA) were 5-10
times greater than that of rV.V-MSA1C-(nSi,nA) and rV.V-MSA1C-
(nSi,A) after the fourth inoculation. One rabbit was inocu-
lated intravenously on day 0 with rV.V-MSA1C-(Si,A) and
reinoculated on days 47 and 68, and the ELISA titer of i.v.
rV.V-MSA1C-(Si,A) was 5 times that of i.d. rV.V-MSA1C-(Si,A).
Also, the ELISA titer of i.v. rV.V-MSA1C-SiA decreased sud-
denly after the third inoculation, possibly because the
antibody neutralized the virus. The data show that rV.V-
MSA1C-(Si,A) and rV.VMSA1C-(Si,nA) can induce significantly
stronger antibody responses against MSA1C-A in rabbits than
rV.V-MSA1C-(nSi,nA) and rV.V-MSA1C-(nSi,A), and that rV.V-
MSA1C-(Si,A) can induce quicker antibody response after a sec-
ond inoculation when introduced intravenously than when intro-
duced intradermally (Figure 9).

(5) Antibody response in mice

Five groups of Balb/c were inoculated i.p. with
1.0×10^8 rV.V-MSA1C(Si,A), rV.V-MSA1C-(Si,nA), rV.V-MSA1C-
(nSi,nA), rV.V-MSA1C-(nSi,A) and WR. rV.V-MSA1C-(Si,A) and
rV.V-MSA1C-(Si,nA) stimulated a level of the C-terminal-
specific antibodies that was 5-10-fold greater than the level
induced by rV.V-MSA1C-(nSi,nA) and rV.V-MSA1C-(nSi,A) after
the third inoculation. The ELISA titer of rV.V-MSA1C-(Si,A)
was about 1:10000 and lasted about 3 months (Figure 10).

Wild type (WR) vaccinia virus coated on a 96-well plate was probed with a serial 10-fold dilution of mouse antibodies raised against rV.V-MSA1C-(Si,A), rV.V-MSA1C-(Si,nA), rV.V-MSA1C-(nSi,nA), rV.V-MSA1C(nSi,A) and WR, and then probed by alkaline phosphatase-labeled goat antimouse IgG. The results showed that the anti-WR virus antibody titers of the recombinant and the WR viruses were almost the same after the second inoculation. Therefore, it appears that all of the mice immunized with the recombinant and WR viruses were successfully infected by the viruses (Figure 10). CBA/J mice were also immunized with rV.V-MSA1C-(Si,A), rV.V-MSA1C-(Si,nA), rV.V-MSA1C-(nSi,nA), rV.V-MSA1C-(nSi,A) and WR viruses. The resulting antibody titers were similar to those of the Balb/c mice although slightly lower (data not shown).

These results indicated that the cell-surface expression of the C-terminal region and the combination of the C-terminal region and the signal and anchor peptides of MSA1 are important in the stimulation of the C-terminal-specific antibody response in rabbits and mice.

rV.V-MSA1C-(Si,A)-induced mouse antibody recognized 190kD protein of parasite in Western blot

Schizont stage parasites resuspended in 1x sample buffer were boiled for 5 minutes and loaded onto a 4-20% Tris-glycine gradient gel, and the proteins were transferred to a PVDF membrane by electrophoresis. The blots were then probed by anti-MSA1C-(Si,A) and anti-MSA1C-A and labeled by alkaline phosphatase-conjugated goat anti-mouse IgG. The 190kD protein

was recognized by anti-MSA1C-A in a Western blot, so we have concluded that the 190kD protein is MSA1. Anti-MSA1C-(Si,A) recognized the 190kD protein (MSA1) as well. (Figure 11) The data demonstrate therefore that the recombinant vaccinia virus correctly expresses the MSA1 fragment in mice.

Indirect immunofluorescence test for parasite

Human erythrocytes infected with the P. falciparum parasite were probed with differing dilutions of the antisera produced by the mice immunized with the four recombinant viruses and the wildtype virus. MSA1C-(Si,nA) and MSA1C-(Si,A) were both positive at a dilution of 1:16, and MSA1C-(Si,A) remained positive at 1:128, while MSA1C-(nSi,nA) and MSA1C-(nSi,A) were both negative at 1:16, as compared to the WR negative control (data not shown).

Invasion Assay

Table 2. Inhibition of P. falciparum invasion of human erythrocytes by antisera (x ± s.%)

	Antiserum dilution in culture			
Antisera	undiluted	1:10	1:100	1:1000
MSA1C-(Si,nA)	54.6±6.7	46.2±9.4	30.9±10.8	28.9±9.7
MSA1C-(Si,A)	62.5±10.2	62.8±5.8	60.4±11.0	45.7±11.9
MSA1C-(nSi,nA)	27.0±10.3	23.7±6.3	20.0±4.6	22.3±3.5
MSA1 C-(nSi,A)	20.2±0.7	12.3±4.9	25.9±6.0	20.6±5.0
WR	11.8±4.0	8.8±8.1	14.2±1.3	3.8±4.9

The assays were performed in duplicate three times each.

Discussion

        The C-terminal region of the MSA1 protein was inserted into the vaccinia virus to investigate the immune response produced by this antigen. Four variations of the MSA1 fragment were used to create constructs which contained neither, one or both of the original MSA1 N-terminal signal sequence and the original C-terminal anchor sequence. Data from immunostaining (date not shown) and Western blot assays (Figure 7) indicated that all four of the recombinant vaccinia virus constructs were expressed in the infected cells. The apparent molecular weight of the MSAIC-(Si,A) construct is less than expected and may be due to proteolytic cleavage of the protein during export. This size discrepency in the recombinant protein is only seen when the anchor region is present in addition to the signal sequence and it is possible that the presence of both of the two regions is necessary for the processing of the protein. The Western blot also reveals multiple bands from the MSA1C-(Si,nA) and MSA1C-(Si,A) proteins and these may be different glycosylation forms. Such a pattern is seen only in the two constructs containing the signal sequence, implying that the protein is glycosylated after entering the Golgi.

        The two constructs with the signal sequence are expressed on the cell surface, whereas the two constructs

without the signal sequence are not. These data indicate that
the signal sequence is necessary for proper export of the
expressed recombinant protein to the cell surface. Further-
more, the data show that the signal region alone is sufficient
for cell surface expression. It is possible that once the
protein has entered the secretory pathway, because of the sig-
nal sequence, there is resultant cell surface expression. The
strength and duration of this cell surface expression appears
to be greater when the protein contains the anchor region in
addition to the signal region and this may be due to
hydrophobic interactions. Data indicate also that the protein
is not secreted externally and thus presumably remains bound
to the cell surface. However, the construct with only the
signal sequence elicits a weaker immune response than does the
construct with both the signal and anchor regions. The anchor
region may be necessary for proper protein conformation which
is necessary for a high immune response.

        Immunofluoresence data illustrate that antibodies
induced by the MSA1C-(Si,nA) and MSA1C-(Si,A) constructs
recognize the parasite in vitro. This indicates that the
recombinant protein expressed by the vaccinia virus is indeed
very similar, if not identical, to the native parasite MSA1 C-
terminus. Antibodies targeting the vaccinia viruses indicate
that all four recombinant virus constructs as well as WR were
administered at approximately the same level. Therefore the
differences of the antibody responses of these four constructs
is due to the primary sequence of the protein, not varying
levels of vaccinia virus. The recombinant protein containing

both the signal and anchor regions elicits the greatest immune response and this suggests that both the signal and anchor sequences are advantageous for optimal expression of the MSA-1 C-terminal region. Furthermore, antibodies to the protein containing both the signal and anchor sequences most effectively inhibit invasion of erythorcytes by parasites. Although 62% inhibition of invasion may seem only a partial inhibition, these invasion assays were conducted with a 3% starting parasitemia, whereas most other studies are done at approximately 0.3%. A higher starting parasitemia may reduce the inhibition because of the large numbers of merozoites released.

This study has shown that the MSA1C-(Si,A) construct expresses a functional protein which possesses the proper signal sequence and the proper anchor sequence for correct cell surface expression. Furthermore, the antibodies produced by mice and rabbits immunized uith the recombinant vaccinia virus are able to block parasite invasion in vitro.

DEPOSITS

The following have been deposited with the American type Culture Collection located at 12301 Parklawn Drive, Rockville, Maryland 20852 pursuant to the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. All restrictions on the availability of the materials deposited will be irrevocably removed upon the issuance of a patent thereon.

Microorganism   **ATCC Designation**

Recombinant Vaccinia Virus   VR-2518
r.v.v-MSA.C (Si,A)

While the invention has been described in its preferred embodiment, it is to be understood that the words which have been used are words of description rather than limitation and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

Claims

    1.   A vaccine comprising an expression vector compris-
ing a nucleotide sequence which encodes for an immunogenic
MSA1 peptide in combination with a mammalian signal sequence
or mammalian anchor sequence.

    2.   The vaccine according to claim 1 wherein said MSA1
peptide is combined with a signal sequence and an anchor
sequence.

    3.   The vaccine according to claim 2 wherein said MSA1
peptide is a carboxy-terminal MSA1 peptide.

    4.   The vaccine according to claim 3 wherein said MSA1
peptide is a carboxy-terminal MSA1 peptide.

    5.   The vaccine according to claim 5 wherein said
carboxy-terminal MSA1 peptide is a 593 amino acid peptide cor-
responding to amino acids 1047 to 1640 of MSA1.

    6.   The vaccine according to claim 6 wherein said sig-
nal sequence is MKIIFFLCSFLFFIINTOC or MKIIF-
FLCSFLFFIINTOCVTHESYOELVKKLEALEDAVLTGYSLFOKEKMVLNE.

    7.   The vaccine according to claim 6 wherein said
anchor sequence is FLGISFLLILMLILYSFI.

    8.   A method of vaccinating a patient against malaria

comprising administering an effective amount of a recombinant vaccinia virus capable of expressing MSA1 peptide after administration of said vaccine to a patient.

9.   The method according to claim 9 wherein said MSA1 peptide is combined with at least one peptide selected from a signal sequence and an anchor sequence.

10.   The method according to claim 10 wherein said MSA1 peptide is combined with a signal peptide and an anchor peptide.

11.   The method according to claim 10 wherein said MSA1 peptide is a carboxy-terminal MSA1 peptide.

12.   The method according to claim 11 wherein said MSA1 peptide is a carboxy-terminal MSA1 peptide.

13.   The method according to claim 12 wherein said carboxy-terminal MSA1 peptide is a 593 amino acid peptide corresponding to amino acids 1047 to 1640 of MSA1.

14.   The method according to claim 11 wherein said signal sequence is MKIIFFLCSFLFFIINTOC or MKIIF-FLCSFLFFIINTOCVTHESYOELVKKLEALEDAVLTGYSLFOKEKMVLNE.

15.   The method according to claim 11 wherein said anchor sequence is FLGISFLLILMLILYSFI.

16. A chimeric peptide sequence comprising:

a) an immunogenic MSA1 peptide;

b) an anchor sequence peptide; and

c) a signal sequence peptide.

17. The chimeric peptide according to claim 16 wherein said MSA1 peptide is a carboxy-terminal MSA1 peptide.

18. The chimeric peptide according to claim 17 wherein said carboxy-terminal MSA1 peptide is a 593 amino acid peptide corresponding to amino acids 1047 to 1640 of MSA1.

19. The chimeric peptide according to claim 16 wherein said signal sequence is MKIIFFLCSFLFFIINTOC or MKIIF-FLCSFLFFIINTOCVTHESYOELVKKLEALEDAVLTGYSLFOKEKMVLNE.

20. The chimeric peptide according to claim 16 wherein said anchor sequence is FLGISFLLILMLILYSFI.

21. An expression vector comprising a nucleotide sequence which encodes for an immunogenic MSA1 peptide and a mammalian signal sequence or a mammalian anchor sequence.

22. The vector according to claim 21 encoding for said MSA1 peptide in combination with a signal sequence and an anchor sequence.

23. The vector according to claim 21 wherein said MSA1 peptide is a carboxy-terminal MSA1 peptide.

24. The vector according to claim 22 wherein said MSA1 peptide is a carboxy-terminal MSA1 peptide.

25. The vector according to claim 21 wherein said carboxy-terminal MSA1 peptide is a 593 amino acid peptide corresponding to amino acids 1047 to 1640 of MSA1.

26. The vector according to claim 22 wherein said signal sequence is MKIIFFLCSFLFFIINTOC or MKIIF-FLCSFLFFIINTOCVTHESYOELVKKLEALEDAVLTGYSLFOKEKMVLNE.

27. The vector according to claim 22 wherein said anchor sequence is FLGISFLLILMLILYSFI.

28. A vaccinia viral vector according to claim 22.

29. A vaccinia viral vector according to claim 23.

30. A vaccinia viral vector according to claim 24.

31. A vaccinia viral vector according to claim 26.

32. A vaccine comprising an immunogenically effective amount of the peptide according to claim 17 administered in combination with a pharmaceutically acceptable carrier, excipient or additive.

33. A DNA sequence corresponding substantially to the

sequence set forth in Figure 2, Figure 3 or Figure 4.

   34. The DNA sequence of claim 33 corresponding sub-stantially to the sequence set forth in Figure 2.

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Fig. 1A

Fig. 1B
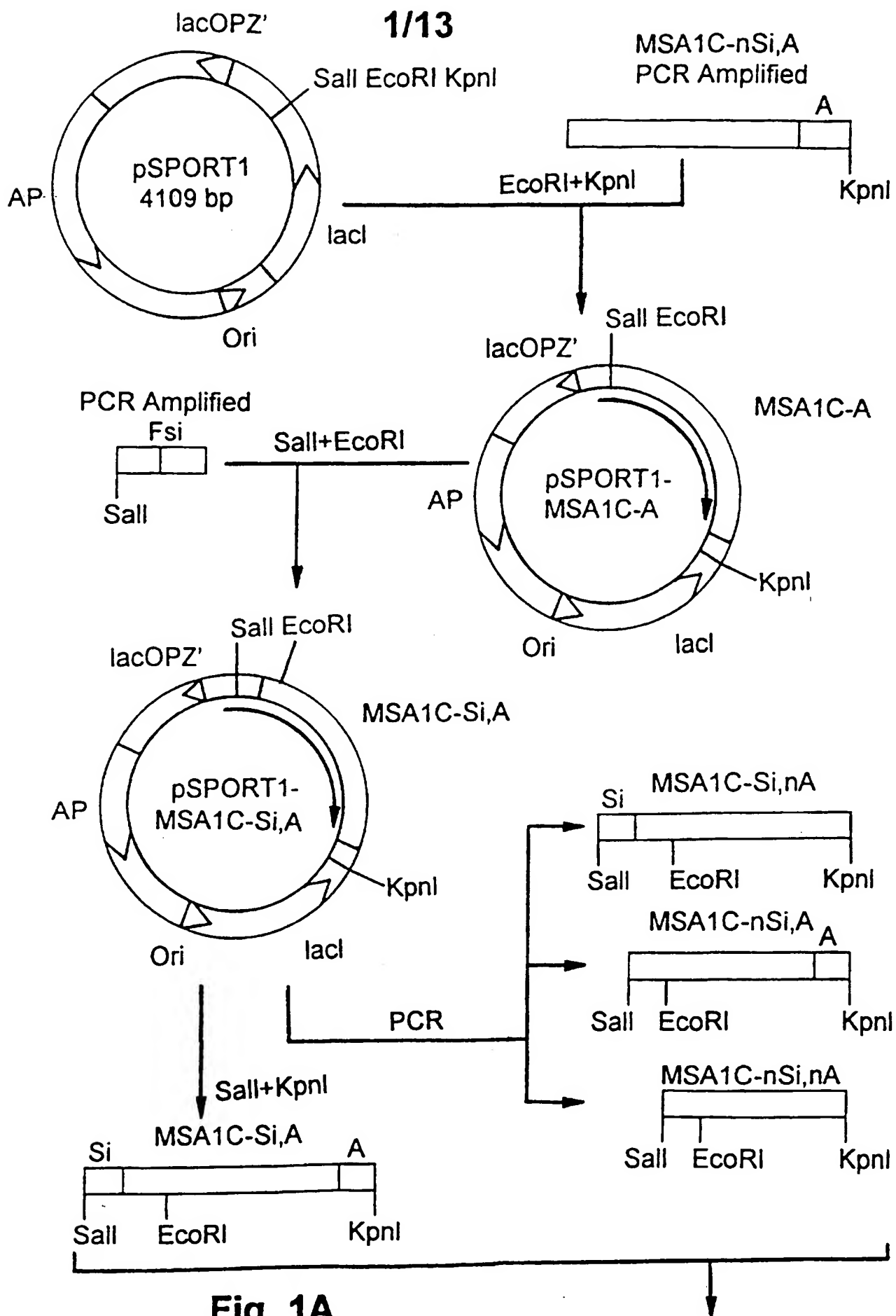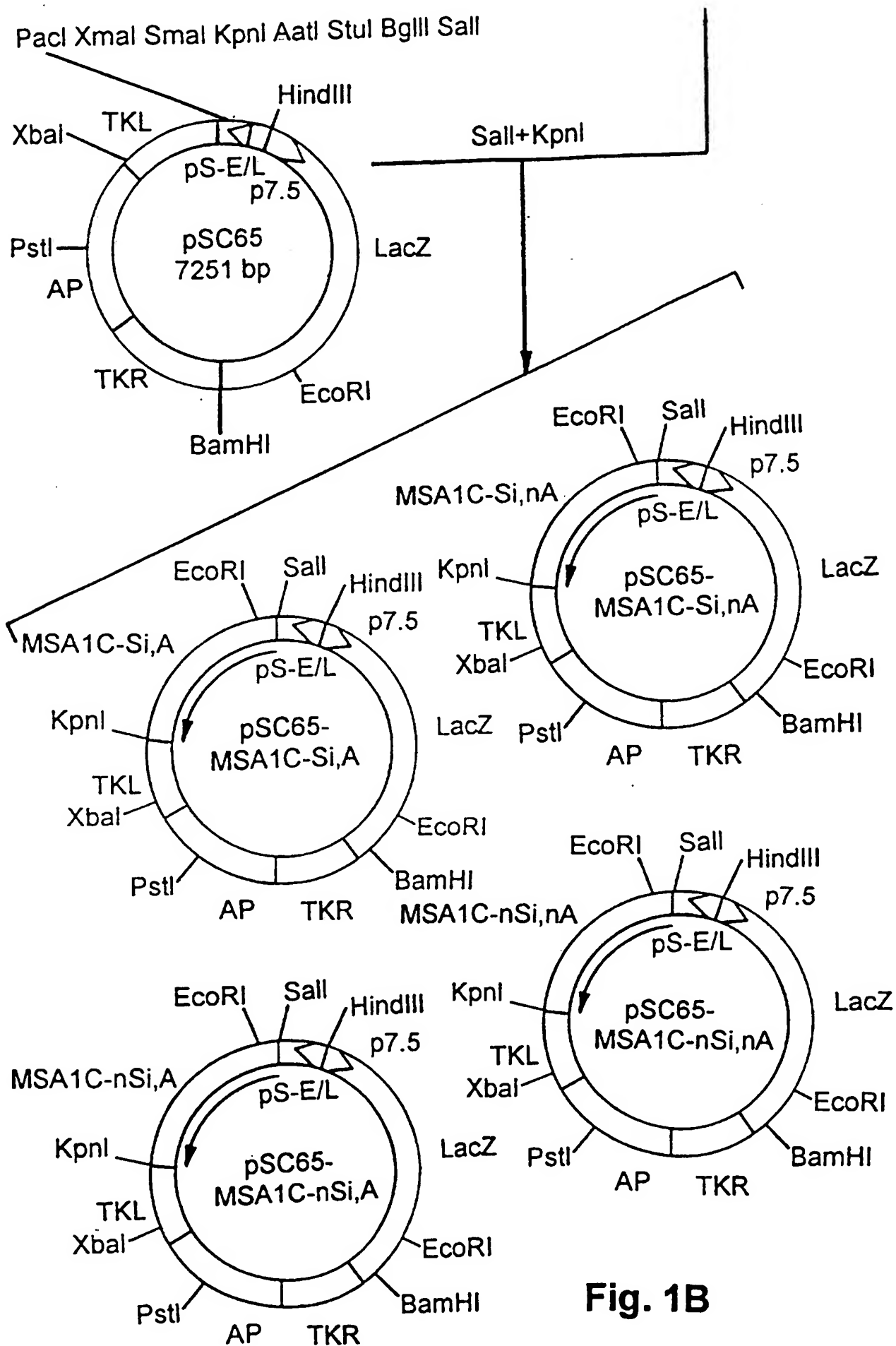
3/13

GENE SEQUENCE OF MSA1C-(Si,A)

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TTTAGAATCA GATTTAATTC CATATAAAGA TTTAACATCA AGTAATTATG
TTGTCAAAGA TCCATATAAA TTTCTTAATA AAGAAAAAAG AGATAAATTC
TTAAGCAGTT ATAATTATAT TAAGGATTCA ATAGATACGG ATATAAATTT
TGCAAATGAT GTTCTTGGAT ATTATAAAAT ATTATCCGAA AAATATAAAT
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GAGAAATACC TTCCCTTTTT AAACAATATT GAGACCTTAT ATAAAACAGT
TAATGATAAA ATTGATTTAT TTGTAATTCA TTTAGAAGCA AAAGTTCTAA
ATTATACATA TGAGAAATCA AACGTAGAAG TTAAAATAAA AGAACTTAAT
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TTTCGTTGGA ATTGCTGATT TATCAACAGA TTATAACCAT AATAACTTAT
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```

Fig. 2

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GENE SEQUENCE OF MSA1C-(Si,nA)

```
ATG  AAGATCATAT TCTTTTTATG TTCATTTCTT TTTTTTATTA TAAATACACA
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TGGTGGATGT GATGCAGATG CCAAATGTAC CGAAGAAGAT TCAGGTAGCA
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```

Fig. 3

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GENE SEQUENCE OF MSA1C-(nSi,A)

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Fig. 4

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GENE SEQUENCE OF MSA1C-(nSi,nA)

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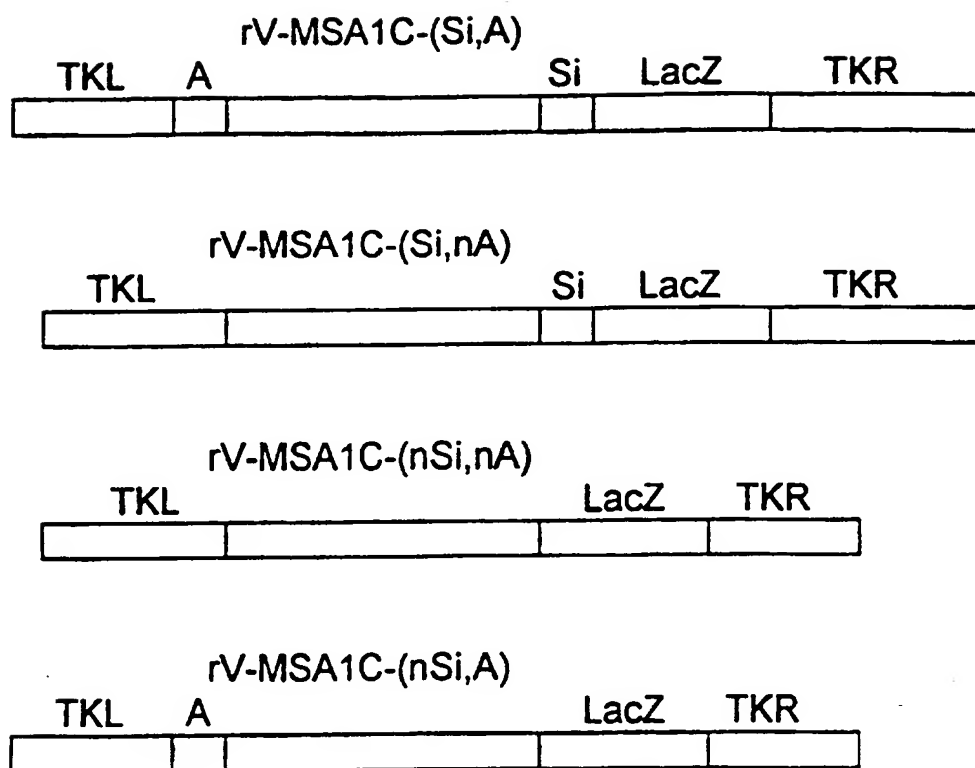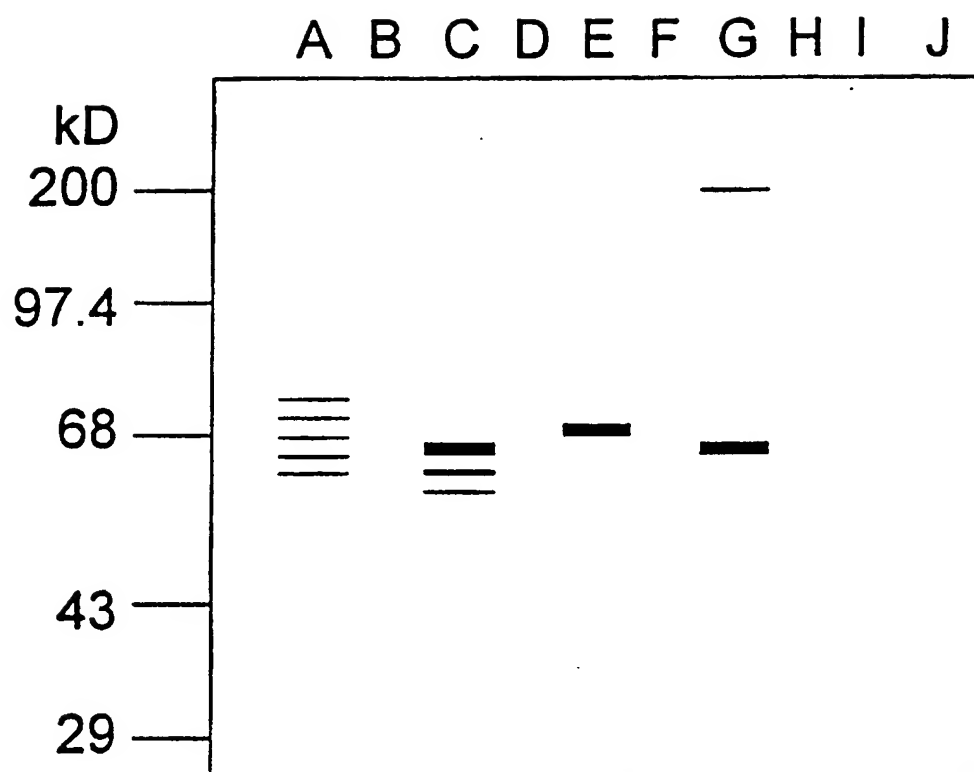
Fig. 5

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rV-MSA1C-(Si,A)

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rV-MSA1C-(Si,nA)

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rV-MSA1C-(nSi,nA)

TKL   LacZ   TKR

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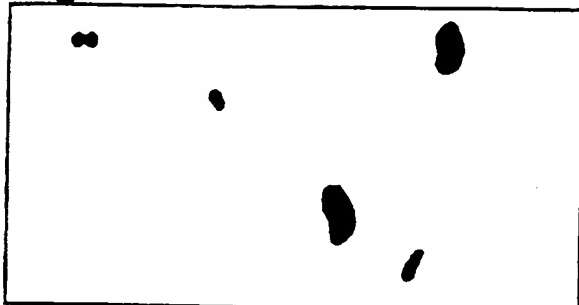
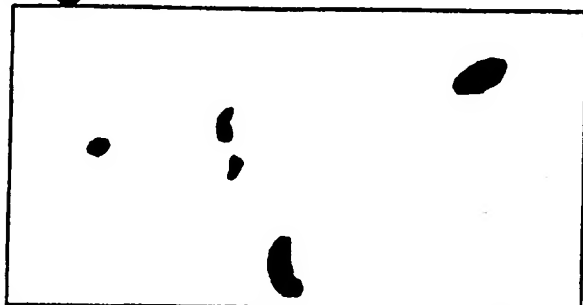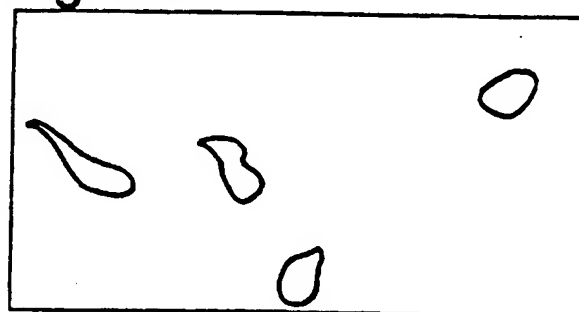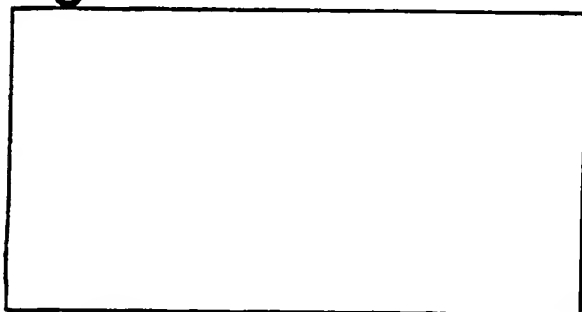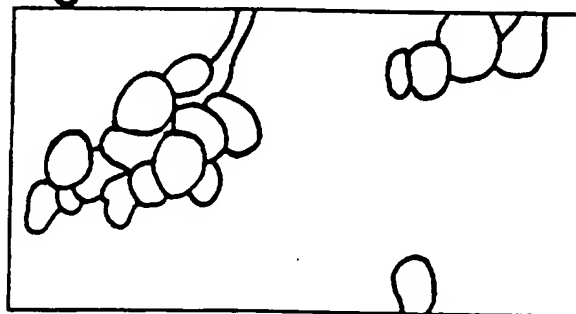TKL   A   LacZ   TKR

Fig. 6

Fig. 7

Fig. 8A

Fig. 8B

Fig. 8C

Fig. 8D

Fig. 8E

Fig. 8F

Fig. 8G
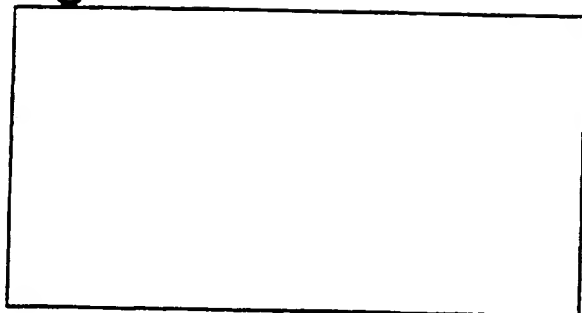
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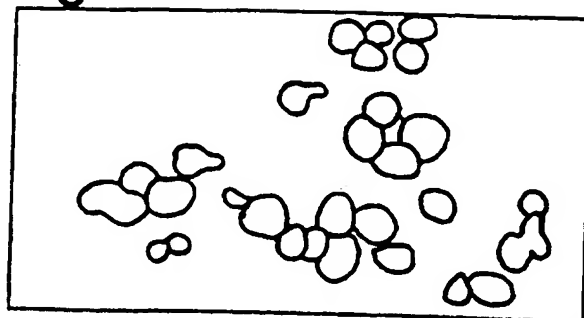
Fig. 8I

Fig. 8J
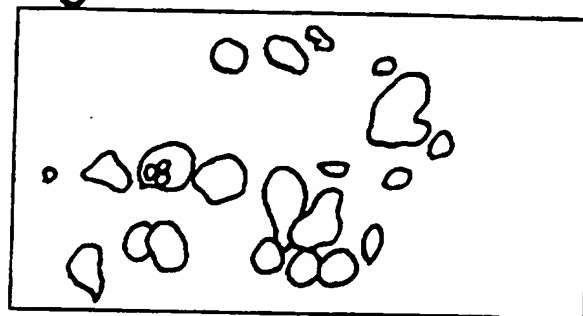
11/13
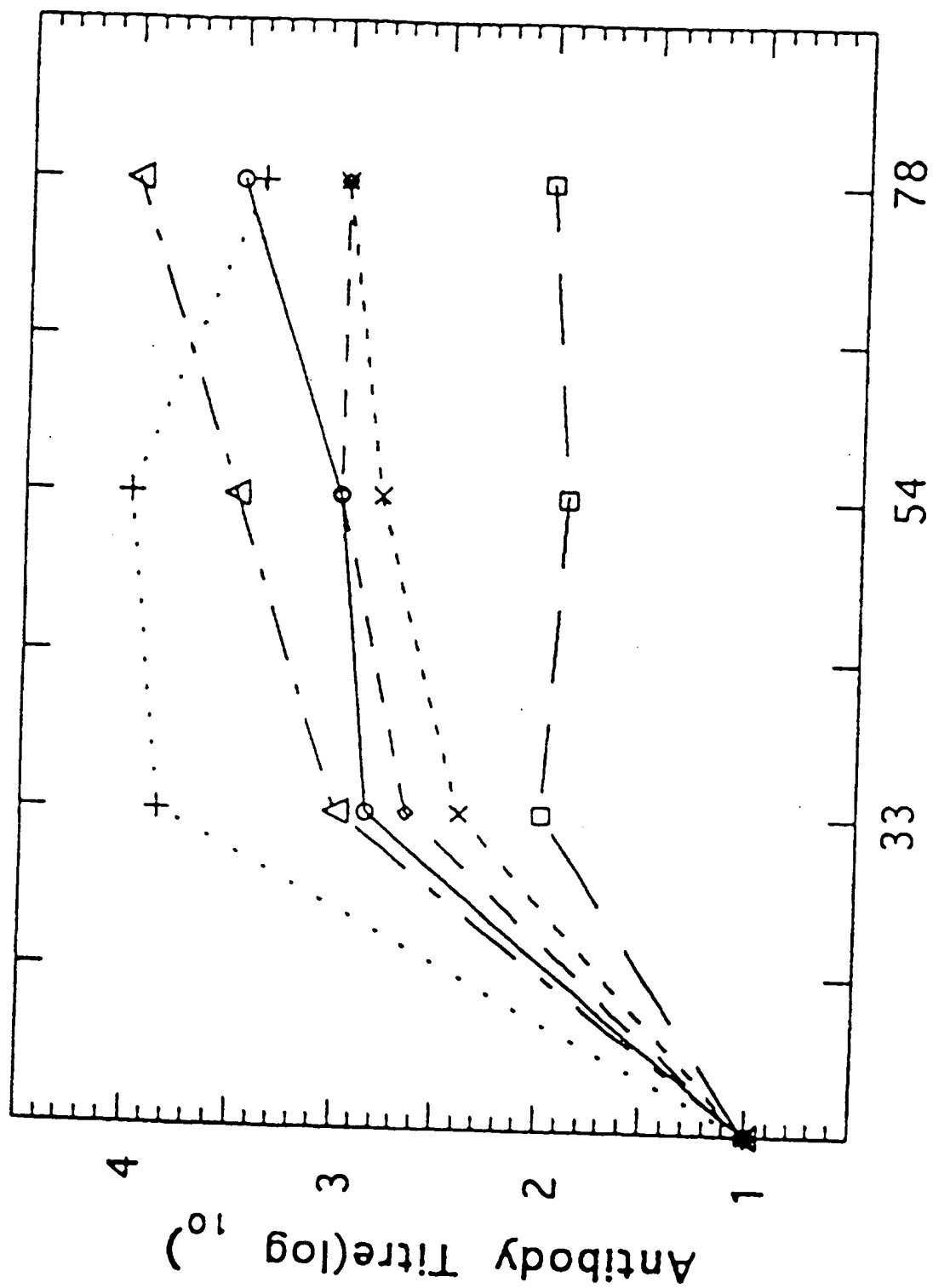
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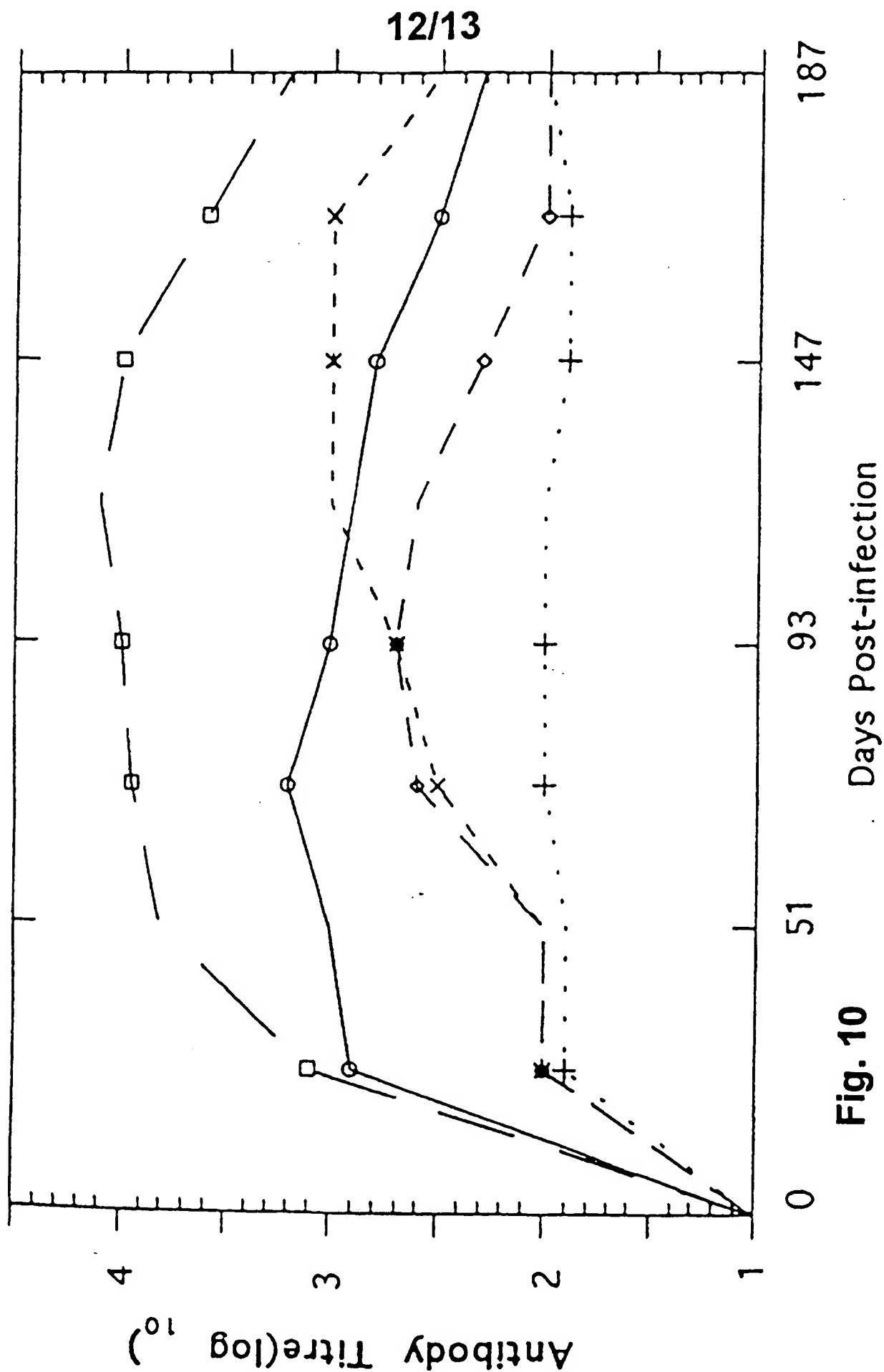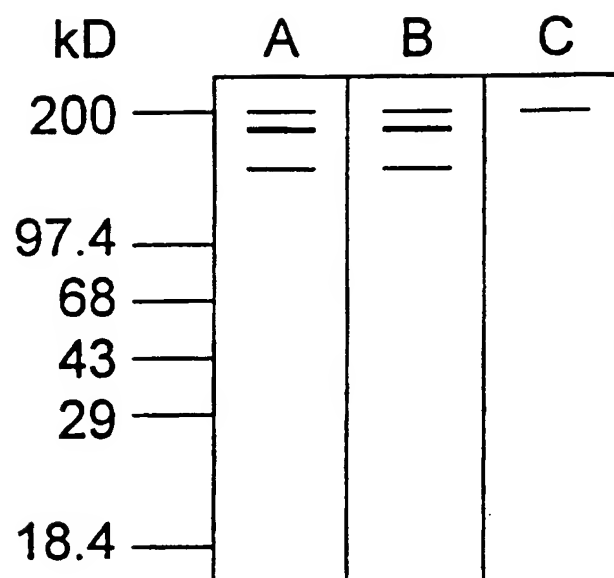
Fig. 10

Fig. 11

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/00; A01N 43/04, 63/00; C12P 21/06
US CL : 424/185.1; 514/44; 435/69.1; 424/93.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/185.1; 514/44; 435/69.1; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,585,268 A (KNAPP et al.) 17 December 1996, column 1, lines 40-65.	1-34
Y	HUI et al. Immunological Cross-Reactivity of the C-Terminal 42-Kilodalton Fragment of Plasmodium falciparum Merozoite Surface Protein 1 Expressed in Baculovirus. Infection and Immunity. August 1993, Vol. 61, No. 8, pages 3403-3411, especially page 3408.	1-34

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 APRIL 1997	2 4 JUN 1997'

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVE NGUYEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/01395

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*